PHYSIOLOGICAL AND MOLECULAR MECHANISMS OF IRON-TOXICITY TOLERANCE IN RICE AND IMPLICATION FOR BREEDING

BY

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DECLARATION

This is my original work and has not been presented for a degree award in any other university.

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DEDICATION

This thesis is dedicated to the memory of my late mother Ruth Onyango.“I have fought a good fight, I have kept the pace and I have finished the race”.

ABSTRACT

Iron toxicity is a widespread nutrient disorder which affects lowland rice mostly in poorly drained fields where reduced iron becomes available at levels exceeding plant's requirements. Different strategies are employed by plants to avoid iron toxicity. However, lack of a clear understanding of adaptation mechanisms used by donor parents and the physiological and molecular factors governing iron-toxicity tolerance mechanisms seriously limits breeding efficiency. The aim of this study was to identify physiological and molecular mechanisms underlying iron toxicity tolerance in rice as well as related QTLs and design breeding strategies that would lead to the development of superior germplasm. Hydroponic and geoponic screening of a diverse set of 17 rice cultivars/varieties obtained from AfricaRice germplasm was done based on split plot randomized complete block design and data analysed using statistical tool for agricultural research (STAR). Based on the preliminary results, four rice varieties contrasting for iron toxicity tolerance were selected for in-depth characterization. Traits associated with Fe-toxicity tolerance were identified through the analysis of morphological (leaf bronzing, plant height, leaf width and length, shoot and root biomass), anatomical (root traits, aerenchyma), physiological (stomatal conductance, photosynthesis, chlorophyll content, fluorescence, cell membrane stability, relative water content, anti-oxidative activities) and metabolic parameters (carbohydrates, proteins, sugars, lignin, suberin). Gene expression changes were assessed by Real Time quantitative PCR (RT-qPCR) using RNA extracted from root and shoot tissues of both control and stressed plants. QTL mapping was conducted in a bi-parental population genotyped with 1,090 SNPs. Among the 22 varieties tested, CK801, CK90, Suakoko8 and IR841 showed the highest tolerance to iron toxicity with limited growth reduction, high number of new lateral roots, low leaf injury and balanced photosynthesis. Detailed analysis of CK801 and Suakoko8 revealed that these two tolerant varieties used different strategies to overcome iron toxicity. Inclusion followed by strong dilution of iron concentration in the cells and efficient reactive oxygen species (ROS) scavenging by anti-oxidants and anti-oxidative enzymes were identified, as some of the additional tolerance mechanisms used by CK801. Suakoko 8 mainly used strong mobilization of carbohydrates at the early stage of the stress period to anticipate metabolite shortage and powerful iron exclusion at the root surface. Iron toxicity tolerance was also expressed through the early down regulation of genes involved in iron uptake and translocation and gradual increments in the upregulation of other genes. In the preliminary QTL analysis, QTLs for root biomass and number of green leaves were detected on chromosomes 2, 4, 9 and 10. Tolerant varieties identified in this study can be used as donors in breeding programs as well as the target traits associated, for the development of superior lowland rice. The advanced Supa x CK801 population (F6) obtained in this thesis will be a valuable tool for multi-location testing and validation of the QTLs reported herein. Selection and characterization of the best performing lines within the populations obtained through the three way crosses with CK801, Suakoko8 and Supa will confirm the efficiency of guided trait combinations as a relevant breeding strategy to achieve stable tolerance to iron toxicity.

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LIST OF ABBREVIATIONS

Bp - Base pairs

$^{\circ}$C - Degree Celsius

Ca - Calcium

CAT - Catalase

Cm - Centimeter

cDNA - Complementary deoxyribonucleic acid

DMA - Deoxymugineic acid

DNA - Deoxyribonucleic acid

dNTP - Deoxyribonucleotide triphosphate

Fe - Iron

Fe^{2+} - Ferrous ion

F^{3+} - Ferric ion

g – Grams

gFW – Gram fresh weight

ha - Hectare

hrs - Hours

H_2O_2 - Hydrogen Peroxide

K - Potassium

Kbp - Kilo base pairs

MA - Mugineic acid

Mg - Magnesium

mg - Milligram

ml - Milliliter

mm - Millimeter

mM - Millimolar

MT - Metric tones

N - Nitrogen

NA - Nicotianamine

$^1 0_2$ - Singlet oxygen

*OsDMAS*1 - Deoxymugineic acid synthase gene

OsFER1-2 - Ferritin genes

OsIR1-3 - Iron regulator genes

OsNAAT1-6 - Nicotianamine amino transferase genes 1 to 6

OsNAS1-3 - Nicotianamine synthase genes 1 to 3

OsIRT1-2 - Iron transporters 1 and 2

OsTOM1 - Mugineic acid transporter

OsYSL - Yellow stripe transporter

P - Phosphorus

PCR - Polymerase Chain reaction

POX - Peroxidase

ppm - Parts per Million

QTL - Quantitative trait loci

RNA - Ribonucleic acid

ROL - Radial oxygen loss

ROS - Reactive oxygen species

Rpm - Revolution per minute

RT - Real time

SOD - Superoxide dismutase

W/V - Weight by volume

Zn - Zinc

% - Percent

µl - Micro litre

µM - Micro molar

µg- Micro gram

µmol-Micro mole

nm-Nano mol

v/v- Volume per volume

w/v- weight per Volume

ACRONYMS

IRRI: International Rice Research Institute

FAO: Food and Agriculture Organization of the United Nations

KARI: Kenya Agricultural Research Institute

MoA: Ministry of Agriculture

CFC: Common Fund for Commodities

USDA: United states Department of Agriculture

SSA-Sub Saharan Africa

WARDA-West Africa Rice Development Association

OECD-Organization for Economic Co-operation and Development

EAC-East Africa Community

FAOSTAT-Food and Agricultural Organization Corporate Statistical Database

NRDS-National Rice Development Strategy

APX- Ascorbate peroxidase

ASA-Ascorbic acid

GSH-Glutathione

ANOVA- Analysis of variance

DMRT- Duncan Multiple Range Test

GLM-General linear model

STAR- Statisticl tool for agricultural research

RGA-Rapid generation advancement

LBS-Leaf bronzing score

PCA-Principal component analysis

MDA- malonaldehide

TPC-Total phenolic compounds

GR-Glutathione reductase

NS-Not significant

FW-Fresh weight

DW-Dry weight

TW-Total weight

RWC-Relative water content

PGO- peroxidase–glucose oxidase

TBA- Thiobarbituric acid

FAA- Formalin Acetic Acid

GL- Green leaves

SLg- shoot legth

LatR-Lateral root

CCI- Chlorophyll content index

PS- Photosynthesis

DSW-Dry shoot weight

TR-Transpiration Rate

DRW-Dry root weight

Chla-Chlorophyll a

Chlb-Chlorophyll b

NADPH-Nicotinamide adenine dinucleotide phosphate

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CHAPTER ONE: INTRODUCTION

Most economies in Sub Saharan Africa (SSA) are supported by agriculture. However, persistent low production levels are prevalent, partly explaining why many rural households are living below poverty line and many are on the brink of starvation and malnutrition (Bahiigwa *et al*., 2005). One of the major crops cultivated in SSA is rice, which covers approximately 128 million hectares of irrigated and rainfed lowland (Maclean *et al*., 2002). However, as many as 100 million hectares of this land show some sort of nutritional constraints to rice growth caused by either deficiencies or toxicities (Brady, 1982; Haefele *et al*., 2014).

Among toxicities, is iron (Fe) toxicity which is well recognized as the most widely distributed nutritional disorder in lowland rice production (Dobermann and Fairhurst, 2000). It occurs in many soils with high active Fe and acidity. In addition, Fe toxicity occurs together with aluminium toxicity irrespective of organic matter and texture (Garrity *et al*., 1986). Factors related to growth parameters, such as age, organic matter and hydrogen sulfide accumulation also generate Fe toxicity in plants (Sahrawat, 2005). Furthermore, reduction of Fe oxides and hydroxides may lead to excessive ferrous Fe in soil (Armstrong, 1979). This toxicity causes decreased mineral uptake and protein content, limits the activities of various enzymes and induces reactive oxygen species (ROS) in plants (Obata *et al*., 1996; Sandalio *et al*., 2001). Further, Fe toxicity interrupts many metabolic processes, leading to severe damage in rice plants. The appearance of iron toxicity symptoms in rice involves an excessive uptake of Fe^{2+} by the rice roots and its acropetal translocation into the leaves where an elevated production of toxic oxygen radicals can damage cell structural components and impair physiological processes

(Becker and Asch, 2005). However, some rice genotypes have shown tolerance in response to Fe toxicity (Becker and Asch, 2005) leading to three types of tolerance mechanisms being proposed in rice: exclusion of Fe in roots; inclusion but subsequent avoidance of Fe via internal distribution; and inclusion along with antioxidant defence (Fang *et al*., 2001; Wu *et al*., 2014). However, detailed knowledge of these genotype-dependent mechanisms is elusive due to lack of targeted studies in rice.

Iron absorption by the roots, and its translocation within the plant require various metal chelators and transporters. Just like all monocotyledonous plant species, rice excretes phytosiderophores to acquire Fe from the rhizosphere, in particular 2′-deoxymugineic acid (DMA) (Quinet *et al*., 2012; Onaga *et al.,* 2016). This strong metal chelator belongs to the mugineic acid family phytosiderophore (MAs) and it solubilizes ferric ions in the rhizosphere. The resulting Fe–DMA complexes are taken up by the roots through transporters from the yellow stripe (*OsYSL*) family (Kobayashi and Nishizawa, 2008). Deoxymugineic acid is synthesized from methionine via nicotianamine (NA), and regulation of this organic acid production depends on iron concentration in the substrate (Quinet *et al*., 2012). Rice possesses three NA synthase genes (*OsNAS1, OsNAS2* and *OsNAS3*) (Inoue *et al*., 2003), six NA amino-transferase genes (*OsNAAT1-6*) (Inoue *et al*., 2008; Widodo *et al*., 2010) and one deoxymugineic acid synthase gene (*OsDMAS1*) (Bashir and Nishizawa 2006; Bashir *et al*., 2006) expected to be involved in DMA biosynthesis. Unlike most graminaceous crops, rice also possesses Fe^{2+} transporters, *OsIRT1* and *OsIRT2* which are able to take up Fe^{2+} directly without reducing the Fe (III) chelates (Ishimaru *et al*., 2006). This could be related to the adaptation of rice to flooded conditions

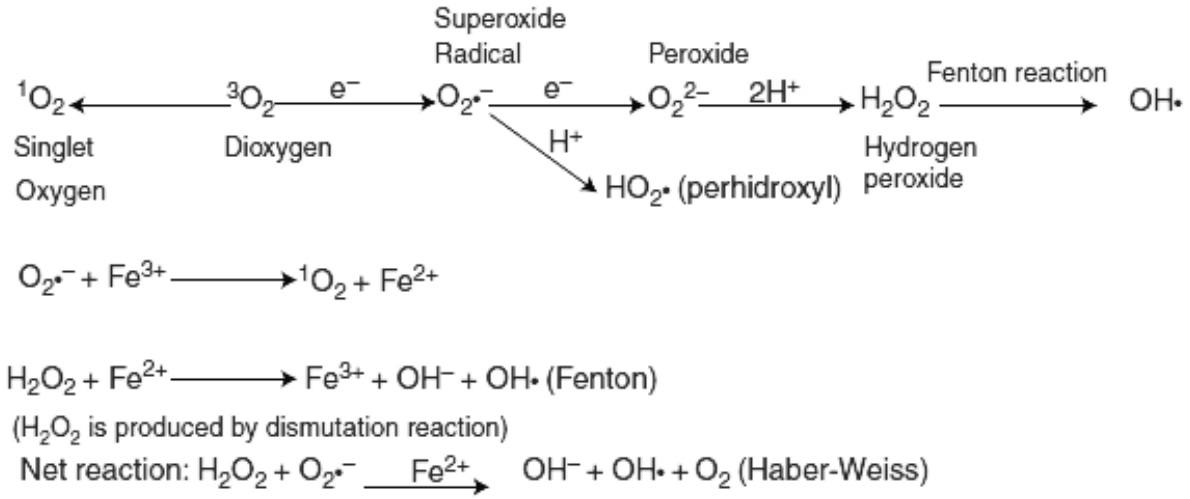
where Fe^{2+} ions are more abundant than Fe^{3+}. NA and mugineic acid are thought to play key roles in the long-distance transport of iron within the plants (Inoue *et al*., 2003).

In plants, more than 80% of cellular iron is localized within chloroplasts in shoots (Marschner, 1995), and these organelles are therefore the first sites of iron toxicity. Excess iron in plants gives rise to a significant increase in cytochrome b6/f content of thylakoids, leading to a higher susceptibility of the photosystem II to photoinhibition and consequently to lower photosynthetic rates and a higher rate of singlet oxygen ($^1O_2$) production (Suh *et al*., 2002). Iron toxicity induces a variety of symptoms, among which the most frequently described is the typical leaf bronzing in rice. Leaf bronzing characteristics in rice brought about by iron toxicity vary on the basis of rice genotypes (Becker and Asch, 2005). According to Sahrawat (1979; 1996; 2000), iron toxicity symptoms are manifested as tiny brown spots starting from the tips and spreading toward the base of lower leaves. As this condition continues, brown spots are observed on the inter-veins of the leaves. In severe cases of iron toxicity stress, the entire affected leaves look purplish brown, followed by leaf drying and death. The roots are also affected by iron toxicity and usually they become short, coarse, blunted and dark brown (Genon et al., 1994). Severe cases of iron toxicity can strongly depress rice grain yield and can lead to losses of up to 100% (Sahrawat, 2003).

Based on the losses observed in rice production systems due to iron toxicity, Becker and Asch (2005) proposed three types of iron toxic conditions. First, due to high content of Fe^{2+} in acid sulfate soils, toxicity symptoms on plants can be observed during the whole growth period. Yield losses in this case range from 40% to 100%. Secondly, in acid clay soils, high Fe concentrations typically occur at around one month or more after transplanting. Under such conditions leaf

bronzing symptoms appear mainly during the late vegetative growth stagewhen rice genotypes are transplanted in the dry season with high vapor pressure deficits. Thirdly, in inland valleys, interflow water containing large amounts of Fe^{2+} can be flushed at the onset of the rainy season from adjacent slopes formed on highly weathered soils into the poorly drained valley bottoms with often sandy soils with low cation exchange capacity. In this case, symptoms can be observed at the early development stages and yield losses can range from 30-70%, but when severe toxicity occurs at the seedling stage, total crop failure can occur.

Various nutrient management strategies to counteract the effects of iron toxicity in rice have been developed. It was recognized that the most promising approach is to use iron toxicity tolerant rice genotypes (Tadano, 1976; Green and Etherington, 1977; Bode *et al*., 1995; Fang *et al.*, 2001; Gallie, 2013). In order to adapt to the varying Fe toxic conditions, rice plants use three major types of tolerance mechanisms. *Type I* refers to exclusion of Fe^{2+} at the root level. Root oxidizing power due to oxygen release or enzymatic oxidation is responsible for the oxidization and precipitation of Fe^{2+} on the root surface, thus avoiding excess Fe^{2+} uptake and translocation into rice shoots (Ando *et al*., 1983; Green and Etherington, 1977). *Type II* refers to the inclusion but subsequent avoidance of Fe^{2+} via internal distribution and storage in a less reactive form. Thus, ferritin is a promising candidate protein as it can accommodate up to 4,000 Fe atoms in a safe and bio-available form (Briat *et al*., 2010). *Type III* refers to inclusion and tolerance to reactive oxygen species (ROS) formed during the Fenton reactions as shown below.



Source: Onaga et al., 2016

Anti-oxidants such as ascorbic acid and reduced glutathione can scavenge ROS to protect plants from oxidative damages (Fang *et al*., 2001; Gallie, 2013) as well as the antioxidant enzymes such as superoxide dismutase, peroxidase and catalase (Fang and Kao, 2000).

Generally, as observed by Audebert and Sahrawat (2000), tolerance of rice plants to higher concentrations of ferrous ion (Fe^{2+}) is assumed to be governed by plant age, its nutritional status and the chemical environment of the root. However, the effectiveness of a given tolerance mechanism depends on the intensity and duration of the Fe stress (Asch *et al.*, 2005). As a matter of fact, physiological and molecular factors controlling iron accumulation within rice plants give rise to a complex network of responses, which are not all clearly elucidated. Quantitative trait loci (QTL) mapping of several traits involved in Fe toxicity tolerance mechanisms has been conducted using different approaches (Wan *et al*., 2003a; Wu *et al*., 2014; Dufey *et al*., 2009, 2015a, 2015b, Matthus *et al*., 2015). However, to date, no reliable diagnostic molecular makers have been reported for use in breeding for Fe toxicity tolerance in rice.

1.1 Problem statement

Crop production in most smallholder farms in SSA is characterized by continuous cultivation coupled with low input use, which results in reduced native soil fertility and productivity. Among the crops widely grown in this region is lowland rice. One of the most important abiotic stress limiting rice production in lowland systems is iron toxicity (Dobermann and Fair-Hurst, 2000;Van Ort, 2018) which occurs as a consequence of the reduction of insoluble Fe^{3+} into soluble Fe^{2+} under anaerobic and low pH conditions (Becker and Asch, 2005; Stein *et al*., 2009).

Iron (Fe^{2+}) acts as a catalyst in Fenton reactions, producing reactive oxygen species (ROS) that are potentially harmful to the cell and that induce oxidative damage in plants (Kao *et al*., 2001; Onaga *et al*., 2016). Therefore, when Fe is absorbed in excess by plants, it can shift the cell redox balance toward a pro-oxidant state, causing alterations in the morphological, biochemical, and physiological characteristics of the plant (Hell and Stephan, 2003) and in the most severe cases, leading to plant death. To maintain balanced levels of Fe that enable optimal plant cell functions while avoiding the harmful effects of Fe-induced oxidative damages, plants must establish tight control mechanisms of cellular Fe levels. Studies shows that excess Fe leads to enhanced generation of ROS in plants due to the strong catalytic power of Fe to generate highly reactive hydroxyl radicals (•OH) from its reaction with hydrogen peroxide (H_2O_2), called the Fenton's reaction (Kao *et al.* 2001). These ROS have been shown to be associated with serious damages in biological systems causing oxidative stress, which could include peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, and ultimately cell death. Thus to counteract oxidative stress, plants have elaborate defense systems of enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase, flavonoids, ascorbate (AsA), glutathione (GSH),

phenolics and carotenoids, which, normally remove or detoxify ROS (Alscher *et al*. 2002). Therefore, it is critical to fully characterize potential donors of iron toxicity tolerance to guide optimal cross combinations to develop tolerant varieties and to accelerate genetic gains in affected soils.

In this study, varieties contrasting inFe toxicity tolerance (including two donors) were screened for various physiological, biochemical and anatomical traits with the aim of gaining a holistic perception of traits underlying rice tolerance mechanisms to Fe toxicity and recommend breeding target traits.

1.2 Justification

Currently about 11 million ha lowland rice areas are prone to temporary submergence in the world (Huke and Huke, 1997). In these areas common soil constraints in rice-based lowlands include salinity/alkalinity (˜ 1.3 million ha), Fe toxicity (˜ 7 million ha), and acid sulfate soils (˜ 2 million ha) (Garrity *et al*., 1986; Akbar *et al*., 1987; Van Bremen and Moormann, 1978). Typical for these characteristics are Oxisols and Ultisols which dominate the landscape of humid and sub humid regions (Onaga *et al*., 2012).

Notwithstanding challenges posed by iron toxicity in rice cultivation, rice still remains the world's most important food crop with a total production around of 600 million ton occupying 11% of the world's total arable land; it supplies 2,808 calories/person/day, which represents 21% of the total calorie supply. It is source of income for more than 100 million householders around

the world (IRRI, 2012). In Africa rice has become a commodity of strategic importance driven by changing food preferences in both urban and rural areas compounded by high population growth. Rice consumption in SSA has in the recent past increased by 5.6% per annum between 1992 and 2015 and projections by USDA suggests rice consumptions in SSA to remain high and continue to increase at 4.5% through the year 2020 an beyond (USDA, 2016).

However, as evidenced by many studies, under Fe toxicity, rice plants display a wide range of responses as part of their strategies to overcome the stress. These strategies include both avoidance and tolerance mechanisms and their efficiency may vary with the type of Fe toxicity occurring in the growth environment, its duration and intensity (Becker and Asch, 2005; Frei *et al.,* 2016). Studies conducted by Dufey*et al*. (2009) reported that in severe cases of iron toxicity, rice plants showed reduction of the water content, and chlorophyll content index an increase in the stomatal resistance, shoot iron uptake and iron concentration of the roots and shoots. The stomatal resistance has also been found to be positively correlated with the leaf bronzing index in iron toxic conditions (Dufey*et al.,* 2009). According to Goicoechea *et al.* (2001) rice plant close its stomata in order to limit the transpiration rate, which may affect the CO_2 exchange rate and related photosynthetic functions and then the plant growth would be reduced. However, in the case of the genotypes used in the study conducted by Dufey*et al.* (2009) stomatal resistance was observed to be a late mechanism of survival after high iron concentration had been reached in the leaf tissue since the more sensitive plants presented both a higher stomatal resistance and a high iron concentration. Several studies have also reported reduction in chlorophyll pigment to be in association with stomatal closure, leading to a reduction of photosynthesis (Dingkuhn *et al.,* 1999; Awal and Ikeda, 2002; Sairam and Saxena, 2000). While a number of reactions and

cellular adjustments are required to circumvent the harmful effects of Fe toxicity most of the genetic studies on this stress have looked at tolerance traits at the plant level (leaf bronzing, plant height, biomass, etc.). It is indeed challenging to evaluate traits at tissue or cellular levels on large populations. Nevertheless, understanding the specific traits contributing to Fe toxicity tolerance in potential donors and singling out individual factors that contribute to these traits could be a first step toward more tailored mapping studies and greater successes in rice improvement for the diverse Fe-toxic environments.

Studies on QTL mapping of traits involved in tolerance mechanisms to iron toxicity has recently been conducted (Wu *et al*., 1997; 1998; Wan *et al*., 2003a, b; 2004; 2005; Fukuda *et al.*, 2012; Wu *et al.*, 2014; Dufey *et al.*, 2015a, b). The genes involved in root to shoot translocation and the transport of Fe to subcellular organelles have also been characterized (Bashir *et al*., 2011a; Bashir *et al.*, 2013b; Ishimaru *et al*., 2009; Ishimaru *et al*., 2012). Yet, resistance to iron toxicity have been reported to be a complex trait which is controlled by several genes which largely depend on the environment as far as their expression is concerned (Wan *et al*., 2004). Even though some knowledge on the genetic and physiological mechanisms underlying iron toxicity tolerance strategies exists, contradicting findings impedes adoption of these findings to date (Gunawardena *et al*., 1982; Akbar *et al.*, 1987; Mahadevappa *et al.*, 1991). In this study it is envisaged that physiological and molecular mechanisms of iron-toxicity tolerance in rice and implication in breeding has been revealed.

1.3 Objectives

1.3.1 Overall objective

The broad objective of this study was to identify physiological and molecular mechanisms underlying iron toxicity tolerance in rice as well as related QTLs and to design breeding strategies for the development and selection of tolerant germplasm.

1.3.2 Specific objectives

1. To identify traits associated with Fe-toxicity tolerance through the morphological, physiological and biochemical characterization of rice varieties/breeding lines.
2. To study differential expression of genes involved in iron nutrition and homeostasis in varieties contrasting for iron-toxicity tolerance.
3. To map QTLs associated with Fe-toxicity tolerance

1.4 Research hypothesis

1. All the rice lines used in this study express similar morphological, physiological and biochemical traits for Fe toxicity tolerance.
2. The rice varieties/breeding lines used in this study contain iron toxicity tolerance responsive genes.
3. There is a strong positive relationship between reported QTLs and Fe toxicity tolerance genes in the population studied.

CHAPTER TWO: LITERATURE REVIEW

2.1 Rice biology, production and constraints

2.1.1 Rice species

Rice (*Oryza sativa*) belongs to the tribe Oryzeae of the family Poaceae (Morishima, 1984). There are 12 genera within the Oryzeae tribe (Vaughan 1994). The genus *Oryza* contains approximately 22 species of which 20 are wild species and two, *O. sativa* and *O. glaberrima*, are cultivated (Vaughan 2003). Both the cultivated species have a relatively small (430 million base pairs) diploid genome (genome AA, 2n = 24) (chang, 2003). Rice is a typical grass, forming a fibrous root system bearing erect culms and developing long flat leaves. It has a semi-aquatic lifestyle, requiring water particularly during the reproductive growth phase (McDonald, 1979). It forms multiple tillers, consisting of a culm and leaves, with or without a panicle. The panicle emerges on the uppermost node of a culm, from within a flag-leaf sheath and bears the flowers in spikelets. The culm consists of a number of nodes and hollow internodes that increase in length and decrease in diameter up the length of the culm (OECD, 1999). Primary tillers emerge from nodes near the base of the main culm and secondary and tertiary tillers emerge sequentially from these. Single leaves develop alternately on the culm, consisting of a sheath, which encloses the culm and a flat leaf blade. The leaf forms a collar or junctura between the sheath and blade and a ligule and two auricles develop on the inside of the junctura and base of the leaf blade respectively. Cultivars can vary widely in the length, width, colour and pubescence of the leaves (McDonald 1979; OECD 1999).

2.1.2 Rice production in Africa

Rice is one of the staple food grown in Africa. According to the Food and Agriculture Organization of the United Nations (FAO, 2010), approximately three billion people – about half of the world population – eat rice every day and this is bound to increase over time. Also, it was reported that growing and processing rice is also the main source of income for an estimated 2 billion people and about 90 % of the world's rice is produced and consumed by small-scale farmers in developing countries (Dufey*et al*, 2012a). Africa alone produces an average of 180.1 million tonnes of rough rice per year (2018) on 150 million ha, equivalent to 19 and 48 percent of the world's total production (Fig. 1a) and rice area, respectively (FAO, 2018). This rise in rice production in Africa is attributable more to area expansion than to yield increase, as yield production per ha remains low (Fig. 1b) (Nwanze *et al.*, 2006; Dufey *et al*., 2012, FAO, 2018). This therefore makes the African countries very vulnerable to increases in the global price of rice as it is expected that the demand for rice will not meet its supply thus forcing Africa to meet its demand through imports from the international markets.

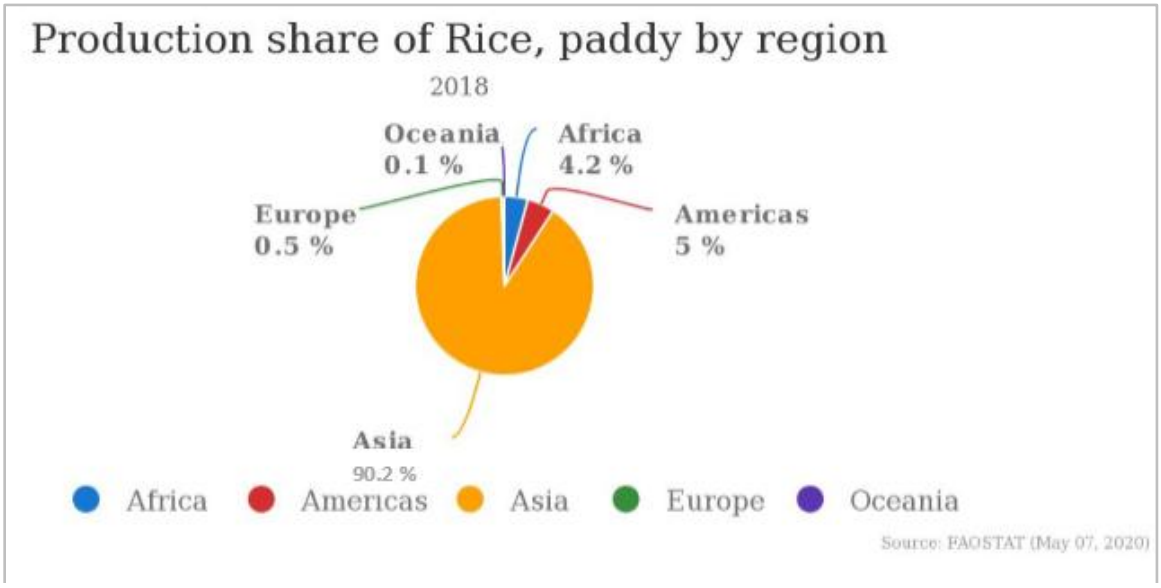


Fig 1a: World rice production (in tonnes)by region

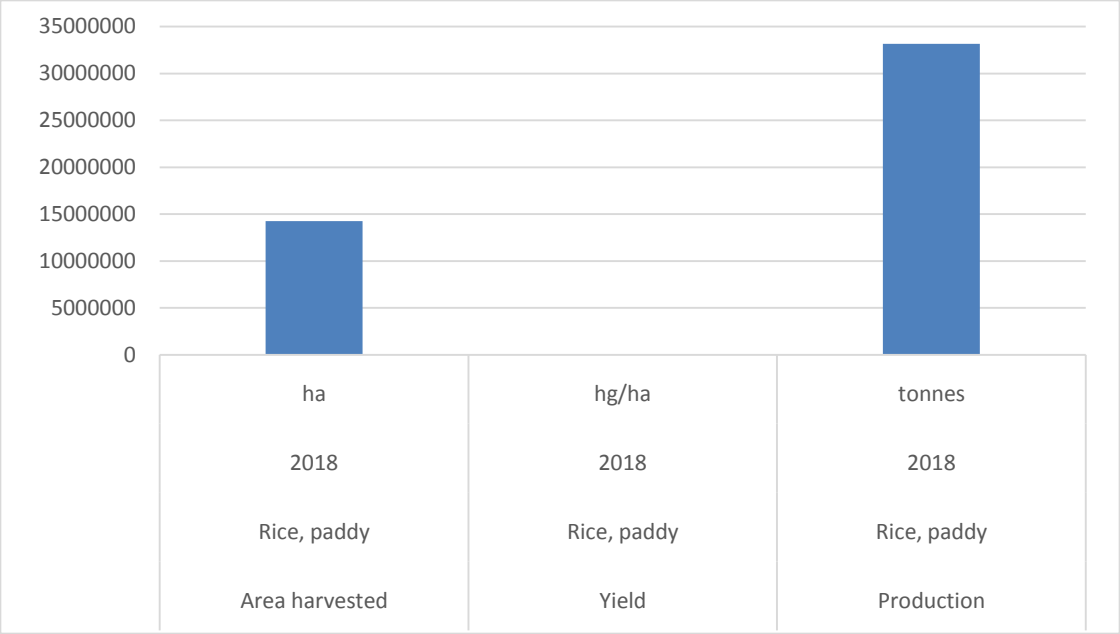


Fig 1b: Rice production in Africa by area, yield and production in the year 2018 (Source FAOSTAT, 2018)

2.1.3 Rice production in East Africa

In SSA has a strong upward trend with Madagascar leading in production followed by Tanzania at a total production of 4 MT and 3MT respectively for the year 2018. Kenyas rice production is the second least after south Africa with a total production of 0.25MT and is in the same production range with Uganda and Rwanda. This trend in production is however expected to grow at a rate of 7.21% over the period of 10 years and all East African countries are expected to increase their annual rice production, resulting in a higher rice production growth rate in East Africa than those of Central Africa and West Africa (Fig. 2) (FAO, 2018).

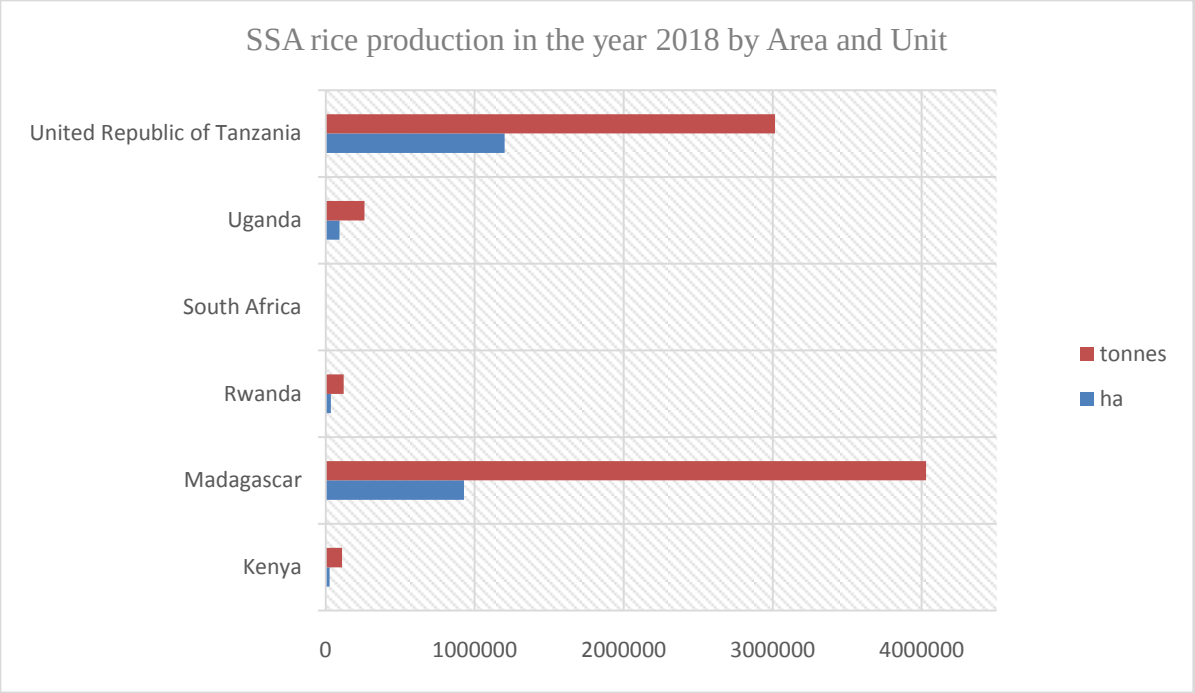


Fig 2: Sub Saharan rice production in the year 2018 by area and unit (Faostat report 2018)

According to USDA 2016 grain report on corn, wheat and rice, east Africa rice consumption is high with a projected high demand by the year 2020 at an increasing demand rate of 54%. This kind of increasing rice consumption can be traced to a combination of factors: population growth, urbanization, changing consumer preferences and economic development. This is a big incentive to increase production and substitute imports which to a greater extent with an exception of Tanzania, supplement domestic production. However, it cannot continue to be business as usual: there has to be a move away from increasing acreage under rice to improving yield production, lack of which renders rice produced in the EAC more costly than that imported (Fig. 3).

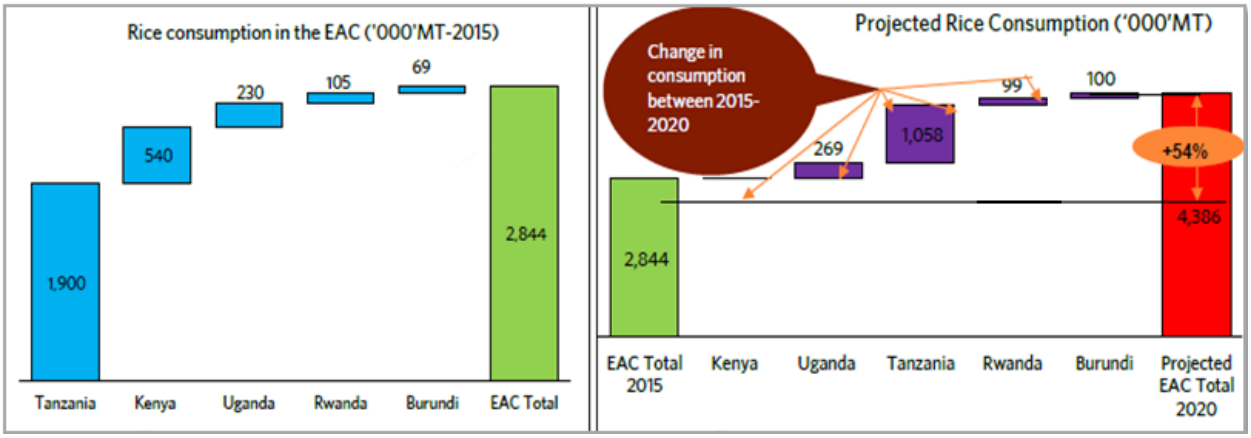


Fig 3: EAC Rice consumption and projected rice consumption between 2015-2020 (Source: USDA, 2016 GRAIN Report)

From the above report none of the EAC countries was self-sufficient in rice as at 2013 except for Kenya. In 2015, the region had an unmet demand of -729,000 MT of which, Kenya had the highest deficit (62%) while Tanzania, the largest rice producer in the region took up 20% of the total lot (*USDA, 2016 GRAIN Report).* This gap between demand and supply has led to importation of rice across EAC with Kenya on the lead (Fig. 4).

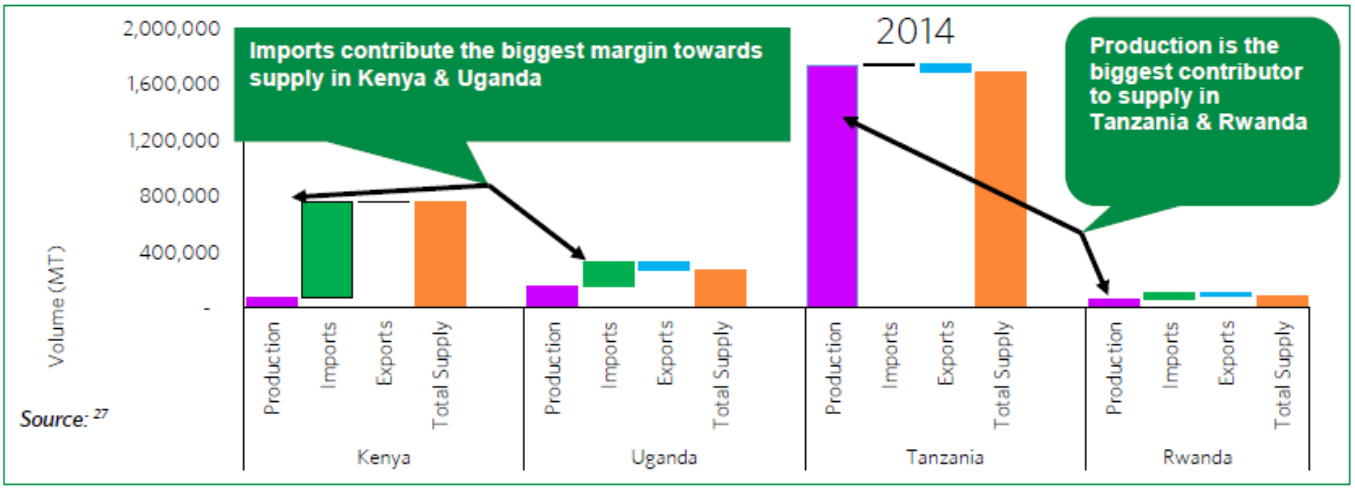


Fig 4: EAC rice production, import, export and total supply for the year 2014
(Source: USDA, 2016 GRAIN Report)

2.1.4 Rice production in Kenya

In Kenya, rice is considered the third most important staple cereal after maize and wheat. However, the country is only able to produce 20% of its national needs (Rice in Kenya (MoA), 2008). Recent years have seen rice grow in importance in Kenya. Per capita consumption, particularly in urban areas, has increased far more rapidly than that for other cereal crops. (CFC_ Rice Sector Development in East Africa, 2012).

Approximately 84% of the rice in Kenya is produced on irrigated land with the remaining 16% being produced under rainfed conditions. The irrigated areas cover approximately 13,000 ha and include irrigation schemes West Kano and Ahero (at 3,520 ha) in Nyanza, Western Bunyala scheme (at 516 ha) and Mwea irrigation scheme (at 9,000 ha) in Central (Rice in Kenya (MoA), 2008; KARI, 2009). Across the years 2009 to 2015, the total rice produced in the country hasn't met the consumption rate (Fig.5) (USDA 2016 Report).

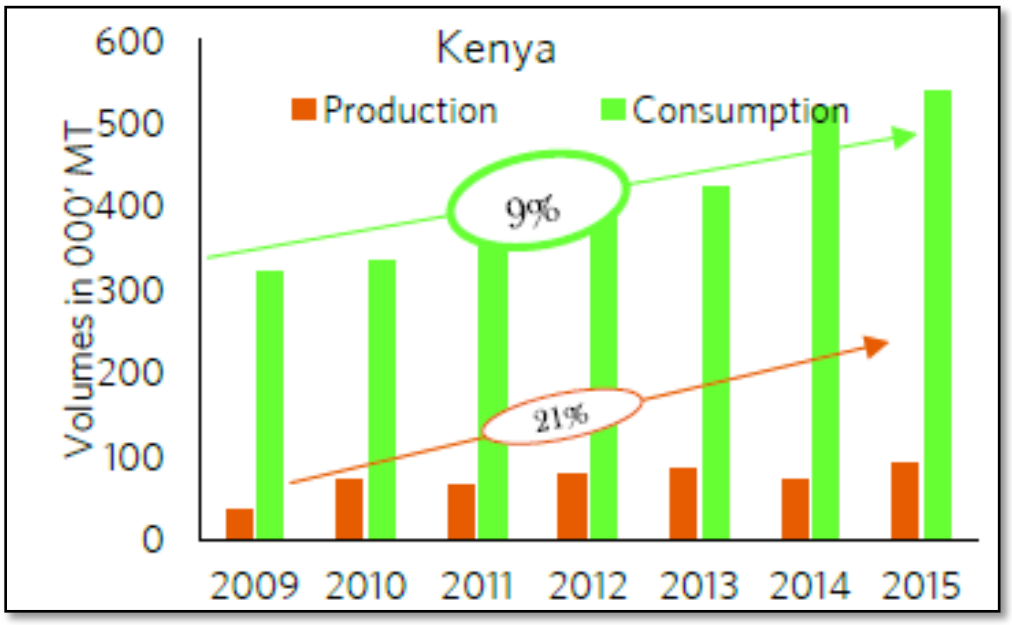


Fig 5: Rice production Vs consumption between 2009 -2015

Source: USDA, 2016 GRAIN Report.

WARDA (2008) estimated Per capita rice consumption in Kenya to be between 10-18 kg per capita per year in figures from 2005. However, annual rice consumption increased in the years after that at a rate of 12% compared to wheat (4%) and maize (1%) (NRDS, 2009). Moreover, the promotion of rice production and consumption in Kenya is anticipated to help do away with the over-reliance on maize as a staple food and therefore to improve rural and urban households' incomes and food security. According to CFC_ Rice Sector Development in East Africa (2012), the area under rice production increased by 111% in the period from 1997 through 2010, while production increased by 54% with an average yield of 3.6 MT/ha.

According to vision 2030, which aims at a vibrant rice sector contributing significantly to improved livelihoods, food security and economic growth, the mission to increase rice production, productivity, value addition and competitiveness through generation, promotion and application of client driven knowledge, information and technologies in a sustainable environment, started in Kenya (Table 1).

Table 1: Rice production in Kenya based on area, yield and consumption between 2008 and 2018 by agro-ecological condition.

Year	Rain fed Upland			Rain fed Lowland			Irrigated			Total		
	Area (Ha)	Yield (tons/ha)	Prod (tons)	Area (Ha)	Yield (tons/ha)	Prod (tons)	Area (Ha)	Yield (ton/ha)	Prod (tons)	Area (Ha)	Yield (tons/ha)	Prod (tons)
2008	2,150	2.72	5,851	3,180	2.76	8,777	12,500	4.7	58,513	17,830	4.1	73,141
2013	3,000	3.11	9,330	4,000	3.20	12,800	18,216	5.1	92,902	25,216	4.6	115,032
2018	4,100	3.70	14,800	5,050	3.76	18,180	26,000	5.6	145,600	35,150	5.1	178,580

Table adopted from the Agricultural Economic Review 2008; National Irrigation Board Strategic Plan 2008 – 2013 and Vision 2030. The figures presented are based on the actual rice production and cultivated area figures for 2008 from four rice producing regions in Central, Coast, Nyanza and Western provinces.

Despite these positive development in rice production across Africa, major challenges ranging from unfavorable land tenure system, unfavorable trans-boundary trade practice, high costs of farm inputs and machinery and low skills/knowledge on rice crop management of abiotic and biotic stresses remains unresolved.

2.2 Rice nutritional requirement for iron and homeostasis

Iron is a key micronutrient for plants, taking part in redox centers of proteins essential for photosynthesis and respiration (Onaga, 2016). Rice has the tendency of taking up more iron than most other plants (Becker and Asch, 2005). In wetland paddy fields, the iron species prevailing is Fe^{2+} which is highly soluble and is easily taken up by the plants (Audebert and Fofana, 2009). After uptake into the root cortex, the reduced Fe^{2+} can enter the xylem after a symplastic passage through the Casparian strip or it may enter the xylem directly *via* an apoplastic bypass (Becker and Asch, 2005), or after root injury resulting from the pulling and transplanting of the seedling. In the xylem, Fe^{2+} follows the transpiration stream in the acropetal long-distance transport. Reaching the leaf apoplastic space, Fe may re-enter the symplast (Stein, 2009).

Mechanisms involved in iron uptake have been well characterized in plants. This metabolic pathway involves two main strategies: the reduction strategy (Strategy I) found in the dicots and monocots not classified as grasses and the chelation strategy (Strategy II-phytosiderophore-based) used by the grass family (Poaceae) (Curie and Briat, 2003; Onaga *et al.,* 2016). Recently, researchers (Quinet *et al.,* 2012; Kobayashi *et al*., 2014; Nozoye *et al*., 2014; Wu *et al*., 2016) have cloned and characterized several genes involved in these iron uptake and transport pathways, including some regulatory transcription factors. Based on Strategy I, Fe^{2+} uptake from the rhizosphere is thought to be principally arbitrated by *OsIRT1*gene (Ishimaru *et al*., 2006),

OsNRAMP1 and *OsNRAMP5*genesin rice (Takahashi *et al*., 2011; Ishimaru *et al*., 2012). Based on Strategy II, rice produces and secretes deoxymugineic acid (DMA), similarly to maize and wheat (Bashir *et al*., 2006). The existence of both strategy I and II in rice could be related to its growth adaptation in flooded conditions.

Deoxymugineic acid is secreted through a specific phytosiderophore (PS) efflux transporter, *TOM1* (Nozoye *et al*., 2011) into the rhizosphere where it binds Fe^{3+}. The Fe^{3+}-DMA complexes are then absorbed into the root mainly through one of the 18 members of YS1-like proteins in rice, *OsYSL15*, in the plasma membrane (Inoue *et al*., 2009; Lee *et al*., 2009). Once in the rice root epidermal cells, Fe in the form of Fe^{2+}-NA or Fe^{3+}-DMA moves towards the central cylinder following the radial symplastic pathway. To enter the xylem vessels, Fe is extruded from the symplast into the apoplastic space through an Fe efflux transporter that was identified in *Arabidopsis* as ferroportin 1/iron-regulated 1 protein (*AtFPN1*/ *AtIREG1*) localized on the plasma membrane of the pericycle cells (Kim and Guerinot, 2007; Morrissey *et al*., 2009; Onaga *et al*., 2016). Alternatively, iron movement from the apoplast requires Fe chelators, nicotianamine (NA), DMA and citrate, which are secreted into the apoplast respectively through the NA efflux transporters *ENA1* and *ENA2* (Nozoye *et al*., 2011), the PS efflux transporter *TOM1* and *OsFRDL1*, a transporter belonging to the multidrug and toxin efflux MATE family (Yokosho *et al*., 2009). Subject on the Fe form and concentration absorbed by rice cells, citrate, DMA or NA could function alternatively or jointly to transport Fe in the xylem. In rice, xylem Fe unloading into the phloem maybe either directly via Fe^{2+} transporters, *OsIRT1*/ *OsIRT2* and *OsNRAMP1*, or as Fe^{3+}-DMA and Fe^{2+}-NA via *OsYSL15*/ *OsYSL16*/*OsYSL18* and *OsYSL2* (Aoyama *et al*., 2009; Kobayashi *et al*., 2014). Further evidence suggests that Fe–citrate could

enter directly into the xylem-associated cells via an unidentified transport system or via a *ZRT-IRT* like transporter after prior reduction of Fe^{3+} into Fe^{2+} by a ferric-chelate reductase (*FRO*) (Roschzttardtz *et al*., 2011). In the phloem, *OsYSL2* is a transporter expressed in the sieve elements that transports Fe^{2+}-NA (Koike *et al*., 2004) while *OsYSL15* transports Fe^{3+}-DMA (Inoue *et al*., 2009). Hence, *OsYSL2* and *OsYSL15* provide rice with a unique opportunity to transport both Fe^{2+}/Fe^{3+}.

2.2.1 Iron toxicity occurrence and management

Iron (Fe^{2+}) is one of the essential micronutrients, which is required by plants in a small amount ranging from 30-150 ppm (Stein *et al*., 2009). It is an essential element that accounts in fundamental reactions such as ribonucleotide and dinitrogen reduction, as well as in the energy-yielding electron transfer reactions of respiration and photosynthesis (Guerinot and Yi, 1994; Hell and Stephan, 2003). In well-aerated soils, Fe is present as ferric hydroxides with low plant availability (Conte and Walker, 2011). However, in most poorly drained colluvial and alluvial soils (sandy and clayey with sediment-derived ultisols), the redox potential (Eh) often falls below +200mV within a few hours after flooding and iron comes into solution as Fe^{2+} (Onaga *et al*., 2013), so that Fe^{2+} concentration increases to thousands mg L^{-1} in the soil solution (Briat and Lebrun, 1999). This phenomenon can induce iron toxicity in plants and occurs especially at actual acid sulfate soils flooded by rain or high tide (Widjaja-Adhi *et al*., 2000), acid clay soils (Alaily, 1998), peat soils (Deturck, 1994) and valley-bottom soils receiving interflow water from adjacent slopes (Sahrawat and Diatta, 1995). In Western Kenya, most soil types tend to be high in active iron and potential acidity, irrespective of organic matter and texture. According to Sahrawat (2008), texture, cation exchange capacity, and organic matter content influence the

concentration of ferrous iron in soil solution, in which iron toxicity occurs. Thus, Fe toxicity is a factor to consider in the rice growing areas in this region (Fig. 6).

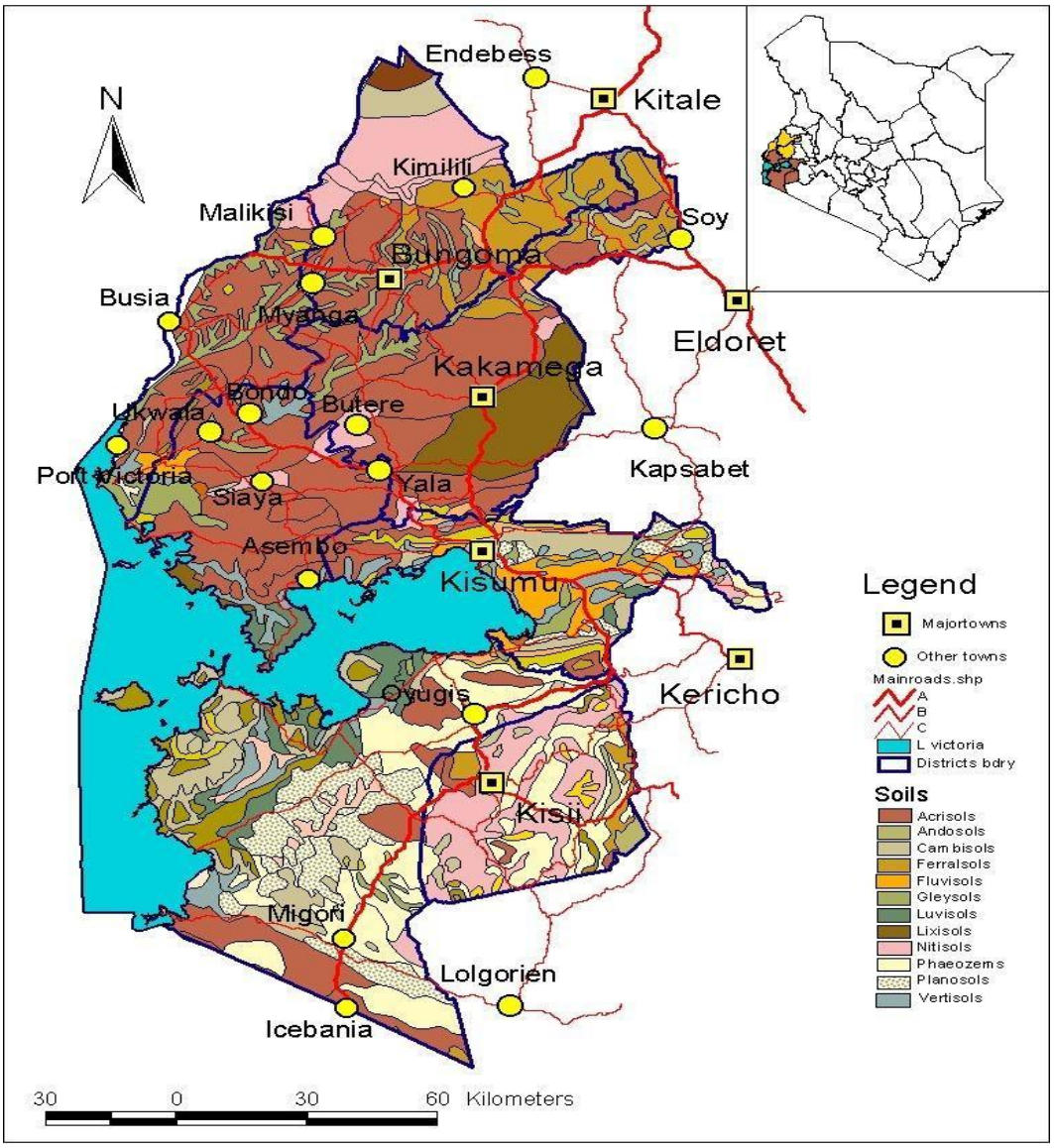


Fig 6: Map of Western Kenya soil. (Adopted from MoA, 2010)

If flooded iron toxic soils are not properly managed before planting or during plant growth, plants tend to absorb Fe^{2+} in excess as it is readily soluble.

In plant tissues, Fe^{2+} participates in Fenton reactions, catalyzing the generation of hydroxyl radicals (·OH) and other reactive oxygen species (ROS) (Briat and Lebrun, 1999; Thongbai and Goodman, 2000). These radicals cause irreversible damage to membrane lipids, proteins and nucleic acids (Becana *et al*., 1998). Eventually they oxidize chlorophyll and subsequently reduce leaf photosynthesis (Pereira *et al*., 2013), thereby leading to yield reductions.

There are several other factors in soil which could favor iron toxicity such as high soil Fe concentration, low soil pH, and waterlogged conditions (Ponnamperuma, 1977b; Van Breemen and Moormann, 1978), nutrient deficiency and nutrient imbalance (Tanaka and Tadano, 1969; Benckiser *et al*., 1982; Yamauchi, 1989), poor drainage, poor root oxidizing power, and application of organic matter that is not easily decomposed (Dobermann and Fairhurst, 2000; Fairhurst *et al*., 2002). Excessive Fe uptake increases polyphenol oxidase enzyme activity resulting in production of high-oxidized polyphenols that cause bronzing.

Several soil management practices have been employed to ameliorate effects of iron toxicity. In inland valleys, planting of leguminous cover-crops and deep-rooting intercrops are practiced (Venus, 2008). Other farmers use water management and a variety of cultural practices (complexing iron with organic matter) in lowland fields (Sahrawat, 2008). However, the use of iron toxicity tolerant rice varieties remains the most recommended option as it is economically viable, environment-friendly, more practical and it can efficiently limit yield losses.

2.2.2 Effect of iron toxicity on growth and development of rice

Iron toxicity is a major problem in rice cultivation. It has been reported from Sri Lanka, India, Vietnam, Malaysia, The Philippines, West Africa, Brazil, Colombia and Madagascar as well as

Burundi and Rwanda (Sahrawat and Singh, 1997). Iron toxicity causes retardation of root growth. The roots become few and short with blunt tip (Yamauchi and Peng, 1993). The range of rice tolerance to Fe toxicity is quite extensive. The critical limit of Fe content in plant tissue is between 300-500 ppm (Sahrawat, 2000). However, other research reported that the critical limit of Fe content in plant tissue is between 500-2,000 ppm (Becker and Asch, 2005; Nozoe *et al*., 2008). Plant could tolerate up to 1,000-2,000 ppm of Fe^{2+} in the soil solution (Asch *et al.,* 2005). Iron toxicity severely limits growth and yield of wetland rice on strongly acid soils with moderate to high amounts of organic matter and reactive iron (Ponnamperuma and Solivas, 1982).

2.3 Mechanisms of iron toxicity tolerance in rice

Rice plants have developed various mechanisms to tolerate or cope with and survive adverse iron-toxic soil conditions and large amounts of Fe^{2+} in the plant tissues. One of these is the control of Fe uptake from the soil which have been studied extensively at the molecular level (Bashir *et al.,* 2010; Bashir *et al*., 2011b; Bashir *et al*., 2013a). Maintenance of a low expression level of Fe uptake genes could be used in the selection of tolerant or adapted rice genotypes to Fe-toxicity given that it is not related to low yield. The relative importance of the tolerance mechanisms to iron toxicity depends on several factors: (1) Oxidation of iron at the root surface (Ando*et al*., 1983); (2) Exclusion of iron at the root surface (Tadano, 1976); (3) Retention of iron in the root tissues (Tadano, 1976) as well as in the shoots, particularly in less photosynthetically active tissues (Audebert and Sahrawat, 2000) and (4) tissue tolerance which is mainly related to oxidative stress control (Mittler, 2004). Based on these different adaptation strategies, Becker and Asch (2005) differentiated tolerant genotypes as “includer” and “excluder” that use respectively “tolerance” and “avoidance” mechanisms.

Rice plants usingexclusion/avoidance strategy exclude Fe^{2+} at the root level and hence avoid Fe^{2+} damage to the shoot tissue (rhizospheric oxidation and root iron selectivity) (Shiono et al 2010). In plants using inclusion/avoidance strategy, Fe^{2+} is taken up into the rice root, but tissue damage is avoided by either compartmentation (immobilization of active iron in "dumping sites" e.g., old leaves or photosynthetically less active leaf sheath tissue) or exclusion from the symplast (immobilization in the leaf apoplast). In theinclusion/tolerance category, riceplants tolerate elevated levels of Fe^{2+} within leaf cells, via enzymatic "detoxification" of generated ROS in the symplast (Becker and Asch, 2005; Onaga *et al*., 2016).

2.3.1 Oxidation of iron at the root surface

Fe^{2+} formed either *in situ* in the soil or *via* interflow, must first pass the oxidation barrier in the rhizosphere before being absorbed by the root (Yukiyoshi and Karahara, 2014). To establish this barrier, molecular oxygen is channeled from the atmosphere through the leaves and stems into the roots *via* a gas-conducting aerenchyma. This aerenchyma forms upon the establishment of anoxic conditions induced by an increased production of ethylene (Kawase, 1981; Yukiyoshi and Karahara, 2014). It involves the degeneration of cortex cells and the subsequent formation of large intercellular lumina (Ando*et al.,* 1983). The rate of iron exclusion by oxidation in the root zone *via* the aerenchyma depends on the phenological stage of the rice plant and on the growth stage of the root system. Young secondary roots and root tips diffuse oxygen at the highest rates into the rhizosphere (Chen *et al*., 1980a). At these growing zones, large quantities of Fe^{2+} can be biologically (microbial) or chemically oxidized, resulting in the formation and accumulation of immobile Fe $(OH)_3$ deposits or iron plaque on the root surface (Chen *et al*., 1980b). The formation of iron plaque on rice roots not only reduces the Fe^{2+} concentrations in the soil

solution, but is also thought to form a physical barrier for further influx of reduced iron (Tanaka *et al*., 1966).

2.3.2 Root membrane selectivity

Reduced iron having passed in the oxidative barrier of the rhizosphere enters the root apoplast. In plants with an undamaged root system, xylem loading requires ions to pass the root cell membranes at the endodermis due to the casparian strip. Reduced iron can be excluded at the root cell membranes (Tadano, 1976), explaining the Fe^{3+} deposition in the apoplast of root parenchymatic cells observed by Green and Etherington (1977). Iron exclusion by root-cell membrane is strongly affected by respiration inhibitors, indicating an active and probably energy-consuming metabolic process (Tadano, 1975). At concentrations larger than 50 mg Fe^{2+} L^{-1}, this reduction process is first impaired and eventually no longer detectable (Tadano, 1976). It is also affected by the rice plants´ nutritional status with deficiencies in Ca and K (Yamanouchi and Yoshida, 1981) and P (Yoshida, 1981) reducing the ability of root cells to exclude iron.

2.3.3 Retention in root and stem tissues

The Fe^{2+} that has entered the xylem stream will follow the transpiration-driven acropetal long distance transport. However, some of this iron may be immobilized and deposited at specific "dumping sites" within the plant (Tanaka *et al*., 1966). The rice plants ability to retain iron inside the root reportedly decreases with plant age (Tadano, 1976). During the further acropetal transport, Fe^{2+} may be immobilized and deposited in stem/leaf tissues (Audebert and Sahrawat, 2000), possibly involving re-oxidation of Fe^{2+}. This "withdrawal" of active Fe may involve the formation of phytoferritin in the xylem and its subsequent storage in stem tissue (Seckbach, 1982; Smith, 1984). The efficiency of this process is likely to be determined by the rate of acropetal Fe^{2+} transport and may not be sufficient to prevent iron influx into the leaves under

conditions of high transpiration such as during exponential growth phase of rice or under dry-season conditions when a high vapor-pressure deficit greatly enhances crop transpiration rates (Asch *et al*., 2000, 2003). The effectiveness may also decline when the storage capacity of stem tissues is saturated, which may occur towards the end of the growth cycle of rice, when the crop was continuously exposed to large Fe^{2+} concentrations and no more leaf tissue is produced (Becker and Asch, 2005).

2.3.4 Retention in the apoplast of the leaf

The apoplastic pH is hypothesized to largely determine the mobility of iron in leaves (Kosegarten *et al*., 1999). An acid apoplastic pH will favor the uptake of Fe^{2+} and pH increase in the apoplast reduces the mobility of Fe and possibly favors Fe^{2+} oxidation (Mengel and Kosegarten, 2000; Nicolic and Römheld, 2001). This is associated with the formation of non-diffusible polymers that reduce the activity of a plasma-membrane bond ferric-chelate reductase (Schmidt, 1999; Lucena, 2000) and involve specific polysaccharides in the cell wall (Yamauchi and Peng, 1995). In plants unable to regulate apoplastic pH or reduce the activity of Fe^{3+} reductase, an uncontrolled accumulation or influx of Fe^{2+} in the leaf can occur (Welch *et al*., 1993).

2.3.5 Symplastic tissue tolerance

Once Fe^{2+} has entered the symplast, it will catalyze the generation of active oxygen species and of various radicals such as superoxide, hydroxyl-radical, and hydrogen peroxide (Marschner, 1995). Prevention of oxidative damage and detoxification of these radicals is vital in alleviating the damage caused by Fe^{2+} and is considered as tissue tolerance (Yamanouchi and Yoshida, 1981; Vose, 1983). Tolerance mechanisms may involve strong binding or incorporation of Fe^{2+} into symplastic structures that allow control oxidation/reduction reactions such as phytoferritin

(Bienfait, 1985). The extent of phytoferritin formation has been hypothesized to be a possible protection mechanism against high concentrations of Fe^{2+} in the symplast (Landsberg, 1996). Radical scavengers such as cytosolic ascorbate (Hu *et al*., 1999) or glutathione (Thongbai and Goodmann, 2000) have also been shown to reduce oxidative stress as induced by Fe^{2+} (Larson, 1988; Thomson et al., 1987). Much of the radical scavenging inside the leaf cell of rice, however, is associated with superoxide dismutase (SOD) isoenzymes. As zinc (Zn) is a component of SOD, Zn application can reportedly increase the iron tolerance of rice while Zn deficiency can produce symptoms that resemble those of iron toxicity (Ottow *et al*., 1982). The activity of SOD results in the formation of hydrogen superoxide (H_2O_2). While this H_2O_2 is a less active oxidizing agent than the radicals, it reduces the activity of SOD (Cakmak, 1988). Hence, to effectively prevent oxidative damage, H_2O_2 needs to be further detoxified by catalases and/or peroxidase (POD) (Gupta *et al*., 1993). The combined SOD-POD enzyme activity has been established to be largely responsible for preventing Fe^{2+}-induced oxidative stress in rice leaves. This mechanism may be of particular importance in situations of seedling toxicity (poorly developed aerenchyma) and especially when rice plants have been injured during transplanting and are exposed to an uncontrolled influx of Fe^{2+} (Yamauchi and Peng, 1995). The genetic variability of SOD-POD activity in the available gene pool is largely unknown to date and has not been used as a screening tool by rice breeders (Becker and Asch, 2005).

2.4 Responses of rice to iron toxicity

Due to the changes in the growth environment, plants have developed elaborate morphological physiological and molecular responses to cope with excess iron in their tissues or in their growth environment.

2.4.1 Morpho - physiological responses

Studies conducted by Dufey *et al*. (2009) reported that in severe cases of iron toxicity, rice plants showed reduction of the root and shoot biomass, water content, and chlorophyll content index, Fv/Fm ratio and an increase in the stomatal resistance, shoot iron uptake and iron concentration of the roots and shoots. According to Dufey *et al*. (2009), this phenomenon is attributed to the existence of free Fe (II) that may generate reactive oxygen species such as superoxide (O^{2}•), hydroxyl radicals (HO•). The free radicals can then oxidize the chlorophyll to reduce its concentration in the cell and a reduction of Fv/Fm which reflects a decrease in the efficiency of the photosystem II. Studies by Bode *et al*. (1995), Peng and Yamauchi, (1993) reported a reduction of the chlorophyll content index and shoot dry weight in rice leaves as effects of iron toxicity.

The stomatal resistance has also been found to be positively correlated with the leaf bronzing index in iron toxic conditions (Dufey *et al.,* 2009). According to Goicoechea *et al.* (2001) rice plant closes its stomata in order to limit the transpiration rate, which may affect the CO_2 exchange rate and related photosynthetic functions and then the plant growth would be reduced. In another report, translocation of iron in the xylem was observed to follow the transpiration stream hence the closure of the stomata was concluded to be a mechanism of avoidance in order to limit the uptake and translocation of iron in the aerial parts (Becker and Asch, 2005). However, in the case of the genotypes used in the study conducted by Dufey *et al.* (2009) stomatal resistance was observed to be a late mechanism of survival after high iron concentration have been reached in the leaf tissue since the more sensitive plants presented both a higher stomatal resistance and a high iron concentration. Several studies have also reported reduction in

chlorophyll pigment to be in association with stomatal closure, leading to a reduction of photosynthesis (Dingkuhn *et al.,* 1999; Awal and Ikeda, 2002; Sairam and Saxena, 2000).

From the above reports, it is evident that physiological consequences of iron accumulation within rice plants give rise to a complex network of responses. Whether these responses are specifically related to plant resistance to Fe toxicity or part of a general stress response are not clearly elucidated.

2.4.2 Molecular responses

According to Quinet *et al*. (2012), response of rice plant to iron toxicity, is regulated by a larger number of genes which were found to be mainly down regulated in the roots during the early stress period and up-regulated in shoots at the later stress period. From this study it was concluded that the expression of genes related to iron acquisition and transport was mainly down-regulated, while the expression of genes related to iron sequestration was up-regulated. Studies by Grotz and Guerinot, (2006); Krämer *et al.*(2007) and Curie *et al*. (2009) reported that a large number of transporters related to iron acquisition, transport and sequestration show a broad specificity for numerous cations, iron and zinc being more especially mediated by the same group of transporters and both elements being complexed by NA and DMA (Curie *et al*., 2009; Ptashnyk *et al*., 2011). Furthermore, Widodo *et al.* (2010), reported the up-regulation of Zn transporter gene *ZIP4* (AK105258) observed in the condition of zinc deficiency which occurred only after 3 weeks of treatment and could not be considered as an adaptative response to Zn deficiencies occurring at the beginning of iron stress. Transcriptomics studies related to plant response to abiotic stresses have over the years focused on short- or long-term response (Chao *et al*., 2005; Chakrabarty *et al*., 2009; Degenkolbe *et al*., 2009), but studies by Quinet *et*

al. (2012) reported the evolution of adaptation strategies to iron excess both on a short (3 days) and long-term (3 weeks) period.

Bashir *et al*. (2014) reported that the expressions of several genes are up-regulated by excess iron in roots and shoots. For example, in roots, members of the cytochrome family, oxidases, alcohol dehydrogenase, a protein kinase, a Zn finger domain-containing protein, and a heavy metal transporter are all up-regulated and many of these genes are also regulated by other stresses. This is the case of cytochrome_P450_family gene, *Os01g0803800,* which is up-regulated by diclofopmethyl (Qian *et al*., 2012); *Os11g0138300,* regulated by ionizing radiation (Kim *et al*., 2012), and a heavy metal transporter which is regulated by excess silicon and rice blast (Brunings *et al*., 2009). One laccase gene that plays a role in lignin formation and two peroxidases (*Os03g0369000* and *Os07g0531400*) are also up-regulated by Fe toxicity (Quinet *et al*., 2012).

2.5 Mapping for tolerance to iron toxicity in rice

In the past 15 years, several quantitative trait loci (QTLs) have been mapped on the rice genome associated with traits involved in mechanisms of resistance to Fe toxicity, under various environmental conditions and using different segregating populations issued from intraspecific crosses (Dufey *et al*., 2009, 2012a; Shimizu, 2009; Shimizu *et al*., 2005; Wan *et al*., 2003a, b, 2004, 2005; Wu *et al*., 1997, 1998; Fukuda *et al.*, 2012; Wu *et al*., 2014) or interspecific (Dufey *et al*., 2012b, 2015a) crosses or using diversity panels (Matthus *et al*., 2015).

QTL mapping has been reported as an effective way to dissect genetic factors underlying phenotypic traits such as Fe toxicity tolerance (Dufey *et al*., 2012a; Fukuda *et al*., 2012; Wan *et al*., 2005). On the other hand, a number of these screening and mapping experiments for tolerance to Fe toxicity in rice have reported somewhat contradictory tolerance rankings. For example, Wu *et al.* (1997) and Dufey *et al.* (2009) while working on *japonica* variety, Azucena screened in 250 mg L^{-1} Fe^{2+} for 4 weeks in hydroponics classified it as tolerant. However, in another experiment carried out by Engel *et al*. (2012) the same *japonica* variety Azucena showed high susceptibility under a pulse stress of 1,500 mg L^{-1} Fe^{2+}. In other studies done by Audebert and Fofana, (2009) on two varieties, WITA 1 and Matkandu from Africa Rice Center and Malaysia, respectively, showed that the two varieties were moderately tolerant in Fe-toxic soils in Korhogo, Ivory Coast, but responded sensitively to Fe stress in Kilissi, Guinea. Yet another *indica* genotype, Pokkali was screened to be sensitive on an acid sulfate soil with chronic toxicity in the Philippines (Gregorio *et al*., 2000), but it showed marked tolerance when treated with an Fe pulse stress of 1,500 mg L^{-1} Fe^{2+} in hydroponics (Engel *et al*., 2012; Wu *et al*., 2014). In other studies, several QTLs have been reported for traits directly or indirectly linked to Fe toxicity resistance Dufey *et al*.(2012 a, b); Wu *et al*.(1997; 1998; 2014); Fukuda *et al*.(2012) and Shimizu *et al*.(2005). Some of these QTLs have been localized at similar chromosomal regions in independent mapping studies, providing high confidence in their implication in the resistance of rice to Fe toxicity. Dufey *et al*., 2015b, Matthus *et al.*, 2015.

Due to the diversity of conditions under which Fe toxicity occurs and the different stress types and intensities, different crop adaptive strategies are required. To overcome the limitations posed by contradictory tolerance rankings, a deeper understanding of the physiological mechanisms of

adaptation to different Fe toxic conditions and the genetic factors behind those mechanisms is required.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Morphological, Physiological and Biochemical characterization

This study aimed at identifying traits associated with Fe-toxicity tolerance and their relationship with the different adaptation mechanisms reported.

3.1.1 Activity 1: Morphological and Physiological responses of seventeen rice genotypes to Fe toxicity

The aim of this activity was to assess genotypic variation associated with iron toxicity in a diverse rice panel.

3.1.1.1 Experimental site

The experiment was conducted in two locations: Africa Rice center, Dar es Salaam, Tanzania and the International Rice Research Institute (IRRI) in Los Banos, Philippines. The former is located 24m above sea level, and longitude 6° 53’15.36” S, latitude 39° 28’13.44”E 32m above sea level and longitude 121.226 S, latitude 14.179 E.

3.1.1.2 Plant materials

Seventeen rice varieties from diverse origin were used in this study (Table 6) Seeds of all varieties were obtained from AfricaRice except Azucena which was obtained from International Rice Research Institute (IRRI). Eight varieties belong to *Oryza sativa* indica, four to *O. sativa* tropical japonica, four to *O. glaberrima* species and two are interspecific *O. sativa* x *O. glaberrima.* All the varieties tested were adapted to lowland ecology except FOFIFA 172 and Azucena which were primarily adapted to upland ecology. Previous studies reported that Azucena is tolerant to iron toxicity while IR64 is moderately sensitive (Wu *et al*., 1997; Dufey *et al.,* 2012a). Suakoko 8 is well-known for its tolerance to iron toxicity (Audebert and Sahrawat, 2000; Sikirou *et al*., 2015) and Supa was reported as sensitive to iron toxicity (Onaga *et al.,* 2013). The other varieties were selected based on preliminary experiments (unpublished data) or recommendations of national partners in Africa.

Table 2: Rice varieties used in this study, species, pedigree and country of origin.

No.	Variety	Species	Pedigree	Origin
1	ARICA 1	*O. sativa indica*	WITA 4 x WAS 161-B-9-3 (NERICA-L 41)	Cote d'Ivoire
2	CK 90	*O. sativa indica*	CK44 x CK801	Guinea
3	CK 801	*O. sativa indica*	CK44 x (CK211 x CK4)	Guinea
4	Suakoko 8	*O. sativa indica*	SIAM 25 x 3*MALUNJA	Liberia
5	IR 64	*O. sativa indica*	Multiparental (8 parents)*	Philippines
6	Supa	*O. sativa indica*	Unknown	Tanzania
7	IR 841	*O. sativa indica*	IR 262-43-8-11 x KHAO DAWK MALI 4-2-105	Philippines
8	Jasmine 85	*O. sativa indica*	IR262 x KHAO DAWK MALI 105	Thailand
9	Azucena	*O. sativa japonica*	Landrace	Philippines
10	FOFIFA 172	*O. sativa japonica*	IRAT 265 57-2 x JUMLI MARSHI	Madagascar
11	X265	*O. sativa japonica*	K28-75-B-1 x KN-1B-214-1-4-3	Madagascar
12	72-5	*O. glaberrima*	Landrace	Liberia

13	TOG 6635	*O. glaberrima*	Landrace	Liberia
14	TOG 6241	*O. glaberrima*	Landrace	Liberia
15	TOG 16771	*O. glaberrima*	Landrace	Guinea
16	NERICA-L-19	Interspecific	TOG 5681 x 3*IR64	Senegal
17	NERICA-L-23	Interspecific	TOG 5681 x 2*IR64 x IR31851-96-2-3-2-1	Senegal

3.1.1.3 Hydroponic screeningusing modified Magnavaca nutrient solution

This screening was done at IRRI, Los Banos, Philippines. First, all seeds were incubated at 50°C for 5 days to break possible seed dormancy. The seeds were then surface-sterilised by soaking in 70% ethanol for 10 minutes thereafter rinsing well with six changes of de-ionised water. The seeds were soaked in de-ionised water for 48h at 32°C and germinated on wet paper towel rolls at 32°C for 48h. After 48 hours, the seedlings were transferred to the screen house (still in thepaper rolls) to acclimatize for a further 1 day before transplanting the seedlings to hydroponic trays containing the modified Magnavaca's nutrient solution (Magnavaca *et al.*, 1987; Adams et al., 2010-Appendix 1b). The experiment was organized in a split plot with randomized complete block design replicated three times. Two treatments (control 0 and 300 ppm $FeSO_4.7H_2O$) were used as main plots. Monitoring of the nutrient solution pH was done every day maintaining it at pH 4.2. Ferrozine and phosphorus (P) assays were done every day to monitor the available $Fe^{2+}$and P in the solution and adjusted as required. Additional tub with nutrient solution but without plants was also maintained to act as a blank for P and Fe quantification. Measurement of morphological and physiological parameters were done before applying iron treatment and thereafter once every week. Full strength nutrient solution was renewed every five days.

3.1.1.3.1 Ferrozine and Phosphorus assay

Ferrozine assay was done to quantify the concentration of Fe^{2+} in the solution on a daily basis. Working stock of 1 mM Ferrozine was prepared and stored in the dark. A sample (10 ml) of the nutrient solution was span at 4000 rpm for 5 minutes and used for Ferrozine assay by measuring the absorbance of the samples at a fixed wavelength of 562nm using BMG SPECTROstar Nano®. Ferrous iron (µM) concentration was calculated as follows:

$$Fe^{2+}\ (\mu M) = \frac{\text{ABS spec value}}{27900 \times DF \times 1000000} \quad \text{.....a}$$

(ABS spec value= absorbance value; DF= dilution factor used to normalize all the samples)

Phosphorus standard stock of 0, 0.5, 1, 2, 5, 10, 50, and 100 µM were prepared in the background of the Magnavaca nutrient solution. The dye (Sulfuric-Ammonium molybdate) (Lowry and Lopez, 1945) was prepared and used to prepare fresh working coloring stock daily for the analysis. Phosphorus quantification was done by measuring the absorbance of the samples at fixed wavelength of 882 nm using BMG SPECTROstar Nano®. A standard curve using ABS readings was made from the standards and the slope of the line was used to quantify the phosphorus concentration available in the culture solutions.

3.1.1.4 Geoponic screening using modified Yoshida nutrient solution

This screening was done at Africa Rice Center, Dar es Salaam, Tanzania. The experimental design was a split plot randomized complete block design with each line repeated three times. The seeds were directly sown in culture pots containing 6 kg of cleaned sieved sand from Ruvu river bank at the rate of two seeds per hole and two holes per culture pot. The pots were regularly

watered using tap water without flooding until seedlings sprout. One week after sowing, 0.5 L of control nutrient solution (*62.5 ml of each nutrient solution plus 0.5 g of $FeS0_4$ [previously dissolved in 100 ml of H20] all mixed in a final volume of 50 L and pH adjusted to 5.7*) was added to each pot once a week. Twelve days after sowing all pots were thinned to two uniform seedlings per hole and gap filling was done using the thinned seedlings. Four days after thinning, Fe treatment was applied twice a week as follows: 62.5 ml of each nutrient solution dissolved in a final volume of 50 L then split into two containers (25 L each). In one container 0.25 g of $FeSO_4$ (previously dissolved in 100 ml of H_2O) was added. The pH of the mixture was adjusted to 5.7 and 0.5 L of the mixture was distributed to each of the control pots. In the second 25 L container, 75 g of $FeSO_4$ (previously dissolved in 100 ml of H_2O) was added. The pH of the mixture was adjusted to 5.7 and 0.5 L of the mixture was distributed to each of the stress pots. After ten weeks of iron treatment, the treatment was stopped and the plants left to grow to maturity. All pots were maintained flooded by adding tap water to maintain 3 cm above the soil surface and control nutrient solution was added once a week. Flowering date was recorded for each variety and harvesting done at physiological maturity.

3.1.1.5 Morphological and Physiological measurements

All the measurements were taken at 7- day interval. Shoot length, root length, leaf length, and leaf width were measured using a meter ruler (cm). Lateral roots and leaf numbers were counted physically per plant once every week. After the third week, leaf bronzing (LBS) was scored based on the visual assessment of the whole plant following the standard evaluation system (SES) for scoring Fe toxicity provided by IRRI (2002) (Appendix 1c). Thereafter, the seedlings were harvested for root and shoot biomass measurements. Roots were rinsed thoroughly using distilled water then blot-dried on a tissue paper before taking the fresh weight (g). Roots and

shoot tissues were oven-dried for 3 days at 70°C after which shoot dry weight and root dry weight were measured and the correlation between dry and fresh root and shoot was determined. The reduction of shoot and root growth was calculated as:

%Relative Shoot Dry Weight = (shoot dry weight in stress)/ (shoot dry weight in control) × 100%;

%Relative Root Dry Weight = (root dry weight in stress)/ (root dry weight in control) × 100%.

Chlorophyll fluorescence was monitored using the pulse amplitude modulation (MINI-PAM, Walz, Effeltrich, Germany) that offers both light and dark adaption of the leaf as well as quantum yield of Photosystem II and leaf temperature, along with a graph of the measuring trace every week.

Leaf stomatal conductance was measured using an automatic porometer (AP 4, Delta-T Devices, Cambridge, UK) between 0900 and 1300 h on the last fully expanded leaf every week on three plants per variety per treatment.

Carbon assimilation, transpiration, and photosynthesis rates were measured using a portable photosynthesis system (LI6400XT, LI-COR, Lincoln, USA) following the manual's guidelines. Internal parameters were fixed at a photosynthetic photon flux density (PPFD) of 1200 µmol m^{-2} s^{-1} by using a LED light source at a reference CO_2 concentration of 400 ppm. The measurements were taken on sunny days between 0900 and 1300 h.

Chlorophyll and carotenoid estimation, the youngest fully expanded leaveswere sampled from plants grown in nutrient solution with and without excess iron and freeze-dried for 3 days.

The dry weights of the samples were determined and the samples placed in 15-ml vials covered with aluminum foil to protect the extracts from light. Ten ml of 95% ethanol were added to the vials before capping. Samples were incubated in the dark for 24 h. Absorbance was read at different wavelengths and chlorophyll and carotenoid concentrations were calculated according to Lichtenthaler and Buschman (2001) using UV-VIS spectrophotometer resolution ranges below.

Chloropyll a (C_a) (µg/ml) =$13.36_{A664.1}$-$5.19A_{648.6}$

Chlorophyll b (C_b) (µg/ml) =$27.43\ A_{648.6}$-$8.12A_{664.1}$

Carotenoid ($C_{(x+c)}$) (µg/ml) = $(1000*A_{470} - 2.13*C_a) - 97.64*C_b) / 209$

Chlorophyll content = µg C_{a+b} /g dry weight

Damage to the plant and photosynthetic apparatus = $(C_{a+b})/(_{x+c})$

3.1.1.6 Statistical analysis

Statistical analysis was performed for each parameter studied based on a Split-plot randomized complete block design model with three replications using Statistical tool for Agricultural Research (STAR) (IRRI, 2013) and R i386 version 3.2.2 (The R Foundation for Statistical Computing Platform, 2015). Associations between parameters were examined using Pearson's correlation analysis except for leaf bronzing score.Duncan Multiple Range Test or Tukey's honest significant difference test was performed to distinguish the different group of varieties for each trait measured.The PCA/biplot analysis was done to help explain inter correlated variables.

3.1.2 Activity 2: Morpho-Physiological, Biochemical characterization and histological observations of four rice genotype contrasting for iron toxicity tolerance

The aim of this activity was to conduct an in-depth characterization of genotypes contrasting for iron toxicity tolerance so as to determine their tissue integrity, metabolic compounds allocation, anti-oxidative enzymes and anti-oxidants activities under toxic Fe conditions.

3.1.2.1 Plant materials, screening and sample collection

This experiment was conducted at IRRI using two tolerant (CK801 and Suakoko 8) and two sensitive rice (Supa and IR64) genotypes selected based on the results obtained in Activity 1. In the screen house, the experiments were set up in a split-plot with randomized complete block design with 3 repsin hydroponics using modified Magnavaca nutrient solution (Famoso *et al*., 2010). Two Fe treatments, control 4.3 ppm HEDTA-Fe and Fe-toxic 300 ppm $FeSO_4 7H_2O$, were used. The pH of the solution was adjusted daily to 4.2. The amounts of iron and phosphorus present in the Fe-toxic treatment were determined daily through Ferrozine and phosphorus assays, adding back into the solution as necessary to maintain the concentration throughout the experimental period.

Root and shoot samples of the four genotypes were collected every 4 days, starting from the second week of seedling growth (before applying the iron treatment) and during a period of 16 days. The sampling scheme was as follows: sampling point 1= 0 days of iron treatment, sampling point 2 = 4 days after application of iron treatment, sampling point 3 = 8 days after application, sampling point 4 = 12 days after application, and sampling point 5 = 16 days after application. All samples were snap-frozen in liquid nitrogen immediately after sampling and transferred to a freezer temperature of -80^0C. The samples were later ground to fine powder using liquid

nitrogen, making sure to avoid thawing of the samples. The ground samples were later weighed and used for the various analyses.

3.1.2.2 Measurement of tissue integrity and metabolic compounds

Membrane stability was assessed using the electrolyte leakage test. Fifteen leaf discs were freshly cut (0.5 cm^2 each) and gently rinsed three times (3 minutes) with distilled water to avoid mechanical damage to the leaf that would increase the amount of ion leakage. They were then floated on 10 ml of distilled water for 16 hours at room temperature under constant gentle shaking. The leaf discs were then incubated in an oven (90 °C) for 2 hours. Ion leakage in the solution was measured using a conductivity meter just before and after heating the samples. The percentage of electrolyte leakage was expressed as the ratio of conductivity before and after heating.

Relative water content *(RWC)* was measured during the last week of Fe treatment since it is destructive. Fully expanded leaves were sampled and immediately weighed to determine the fresh weight (FW). Leaves were then immersed in petri dishes containing 5 mM $CaCl_2$ overnight at room temperature in a closed box to reduce vapor loss. The next day, leaves were removed and quickly blot-dried on a tissue paper then weighed to determine turgid weight (TW). Dry weight (DW) was determined after oven-drying the leaves at 74 °C for 3 days. The RWC of the leaf was calculated using the formula RWC = [(FW – DW) / (TW – DW)] x 100.

Tissue iron: The determination of Fe content in plant tissues was done on the four uppermost and the three lower leaves of 30 day-old rice seedlings screened in hydroponics under toxic iron

levels (300 ppm). The shoots were detached from the roots at the culm base and weighed to determine fresh biomass. Roots were then rinsed thoroughly using distilled water, blot-dried on tissue paper, and their fresh weight determined. The fresh roots were incubated for 3 hours at 25°C in 45 mL of a solution (pH 6.5) containing 0.27 M sodium citrate ($Na_3C_6H_5O_7.2H_2O$) and 0.11 M sodium bicarbonate ($NaHCO_3$), with the addition of 3.0 g sodium dithionite ($Na_2O_4S_2$) to remove the iron plaques on the roots. The washed roots and shoots were oven-dried at 70°C for 3 days and the dry weight determined. The dried samples were finely ground in a stainless electric grinder. Subsamples of 300 mg of ground samples were ashed in a furnace at a temperature of 325°C until all smoke dissipated. Complete ashing was then done overnight at 490°C. The samples were cooled at room temperature and the ashes reconstituted with 50 ml 1N HCl. The dissolved ashes were centrifuged at 10,000 g for 10 minutes and intracellular iron determined in the extracts using an atomic absorption spectrophotometer (Shimadzu, model AA60IFG).

Soluble sugars*:*Two hundred (200) mg of each freeze-dried shoot and root samples were finely ground and put in 15-mL falcon tubes with screw caps. Seven ml of 80% ethanol was added to the samples vortexed at a maximum speed for 2 s and centrifuged at 3,500 rpm for 5 minutes. The supernatantswere collected into 25 ml volumetric flasks. The process was repeated twice, adding 3 ml of 80% ethanol at the third time. The final volumeof the sample was adjusted to 25 ml using 80% ethanol and the volumetric flask was covered using parafilm to avoid evaporation (Fales et al., 1951). Sample assays were prepared with a different proportion of sample extract for the shoot and root tissues. For root samples, 0.1 ml of sample and 1 ml anthrone were used. For shoot samples, 0.05 ml of sample were mixed with 0.05 ml 80% ethanol and 1 ml anthrone. All the samples were incubated in a water bath at 95 °C for 10 minutes and

the reaction stopped in ice bath. All samples were vortexed at maximum speed for 2 seconds before absorbance readings were made at 620 nm using BMG SPECTROstar Nano®. Total soluble sugar was expressed as the percentage by weight of glucose per unit dry weight of the sample used in the extraction.

Starch content*:*Dry residues from the sugar assay were transferred into clean 15 ml falcon tubes together with 10 mg of starch standard and 2 mL of acetate buffer. All the samples were incubated in a water bath for 3 h at 95°C with regular mixing every 20 minutes. The mixture was cooled to 55 °C and 1 mlamyloglucosidase in acetate buffer was added immediately. The samples were vortexed at maximum speed for 2 seconds and incubated for 24 hours at 37°C, after which the samples were centrifuged at 3,500 rpm for 10 minutes and the supernatant transferred to a 25 ml volumetric flask. Solid residues were cleaned using 3 ml of Nano pure water vortexed and centrifuged at 3,500 rpm for 10 minutes. The supernatant was transferred into a volumetric flask and the extract volume toped up to the 25 ml using Nano pure water. The flask was covered with parafilm, while the starch sample was used to prepare the standards.

Sample preparation for the assay was done by mixing 0.6 ml of the extractant with 3 mlperoxidase–glucose oxidase/o-dianisidine (PGO) enzyme color reagent. The mix was vortexed at maximum speed and incubated in the dark for 30 min (Draeger and Ziegehorn, 1988). Absorbance of the samples was read at 450 nm using BMG SPECTROstar Nano®. Starch concentration was expressed as gram per dry weight of sample used for soluble sugar extraction.

Lipid peroxidation levels were assessed by measuring malondialdehyde (MDA) concentration as described by Hodges *et al*. (1999). Malondialdehyde was extracted from 200 mg of finely ground leaf and root samples by adding 2.0 mlof extractant buffer (4°C, 80:20:0.01 v/v/w ethanol: water: trichloroacetic acid) to the ground sample and homogenizing for 1 minutesfollowed by vortexing at maximum speed for 2 seconds. The samples were centrifuged at 4 000 g, 4 °C for 15 minutes and 1.5 ml of the supernatant was carefully collected into clean micro-tubes. The aliquot of the supernatant was divided into two tubes, 0.5 mL was added into a tube containing 0.5 ml (+) TBA (Thiobarbituric acid) reagent, the other 0.5 mL, into a tube containing 0.5 ml (-) TBA reagent. The tubes were vortexed at maximum speed for 2 seconds and incubated at 95°C for 25 minutes. The reaction was stopped on ice. Absorbance was read at 440, 532, and 600nm using BMG SPECTROstar Nano®. Malondialdehyde concentration was calculated from the differences in absorbance at 440, 532, and 600 nm using the extinction coefficient 157 mM^{-1} cm^{-1}, as shown in the following equations:

$[A_{532(+)TBA} - A_{600(+)TBA}] - [A_{532(-)TBA} - A_{600(-)TBA}] = A$

$[A_{440(+)TBA} - A_{600(+)TBA}] * 0.0571 = B$

MDA equivalents (nmol mL^{-1}) = $[(A - B)/157{,}000]10^6$

MDA values were expressed relative to fresh matter used for the assay as nmol ml^{-1} g^{-1}.

Total phenolic content *(TPC):*The quantification of TPC followed the protocol of Ainsworth *et al*. (2007). The sample extract remaining from the MDA quantification was used. Aliquots of 100 µl and/or gallic acid standards were added into a tube containing 200 µl of 10% (v/v) Folin-Ciocalteau phenol reagent. The tubes were vortexed at maximum speed for 2 seconds and incubated at 25 °C for 6 minutes in the dark and then 800 µl of 700 mM Na_2CO_3 was added. The

mix was vortexed at maximum speed for 2 seconds, and incubated at 25 °C for 2 h in the dark. Absorbance values were read at 765 nm using BMG SPECTROstar Nano® and total phenolic content was imputed based on the standard curve of gallic acid. The results were expressed as gallic acid equivalents gFW^{-1}.

Protein content*:*Frozen 200 mg finely ground leaf and root samples were used for protein extraction. The samples were kept on ice to inactivate any enzyme activity and 2 ml of cold extraction buffer was added immediately before thawing. The samples were vortexed at maximum speed for 2 seconds, agitated at 4°C for 10 minutes and then centrifuged at 10,000 g, 4°C for 10 minutes. The crude extract was collected in a clean, labeled tube and the residue discarded (Tomlinson *et al*., 2004). This crude extract was then subjected to dialysis for 24 hours with constant agitation at 4°C, changing the dialysate every 6 hours to ensure maximum purification of the proteins. Purified protein was quantified following Bradford (1976).

3.1.2.3 Analysis of anti-oxidative activities and anti-oxidants

Purified protein samples were normalized to 10 µg ml^{-1} for all enzyme kinetics and analysis was done following the procedure of Brand-Williams *et al.* (1995).

Superoxide dismutase *(SOD)* activities were determined using 185 µl of 50 mM potassium phosphate buffer (pH 7), 25 µl of 1mM xanthine, 25 µl of 0.1 mM cytochrome C, 10 µl of the sample extract, and 5 µl 0.5 (U) xanthine oxidase were used per sample. Absorbance was measured at 550 nm using BMG SPECTROstar Nano®. Enzyme activity was calculated using the following equation:

SOD activity= ((slope of blank)/ (slope of sample))-1

The activity of the enzyme was expressed in units/mg of protein.

Ascorbate peroxidase *(APX)* activity was determined using 200 µl of 50 mM potassium phosphate buffer (pH 7), 25 µl of 5 mM ascorbic acid, 10 µlof the sample extract, and 15 µl of 10 mM H_2O_2 per sample. Absorbance was measured at 290 nm using BMG SPECTROstar Nano®. Enzyme activity was calculated using the corresponding molar extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

Catalase *(CAT)* activity was determined using 230 µl of 0.1 M phosphate buffer (pH 7), 10 µl of sample extract, and 10 µl of 1 M H_2O_2 per sample. Absorbance was measured at 240 nm using BMG SPECTROstar Nano®. Catalase activity was calculated using a molar extinction coefficient of 39.4 $M^{-1}cm^{-1}$ based on H_2O_2 destroyed/min/mg protein.

Glutathione reductase*(GR)* activity was determined using 225 µl of 0.2 M Tris/HCl buffer (pH 7.8), 10 µl of 5mM NADPH, 10 µl of sample extract, and 5 µl of 0.05 mM oxidized glutathione per sample. Absorbance was measured at 340 nm using BMG SPECTROstar Nano®. GR activity was calculated using the corresponding molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

Peroxidase *(POX)* activity was determined using 125 µl of 50 mM sodium acetate buffer (pH7), 100 µl of 0.05% guaiacol, 10 µl of sample extract, and 15 µl of 10 mM H_2O_2 per sample.

Absorbance was measured at 470 nm using BMG SPECTROstar Nano®. POX activity was calculated using the molar extinction coefficient of 26.6 $mM^{-1}cm^{-1}$.

3.1.2.4 Histochemical observations

Sampling and fixing of tissues*:*The main root and youngest fully expanded leaf and main stem of 28day-old rice seedlings grown under excess Fe^{2+} (300ppm) were harvested and carefully stacked in tubes containing 100 mlFormalin Acetic Acid (FAA) and alcohol (Johansen, 1940) to fix the tissues. The fixative (FAA) was prepared using 50 ml of 95% ethanol, 5 ml of acetic acid, 10 ml of 37% formaldehyde, and 35 ml of distilled water. The samples were kept at 4°C before being sectioned.

Observation of aerenchyma and quantification*:*Cross-sections of the main root (root cap: 0-5 mm, mid root: 10-15 mm, root base: 15-20 mm), leaf sheath, and stem were done manually and observed using a microscope equipped with a camera

Observation of suberin and lignin deposits in the cell membranes of roots, leaves and stems*:*Sections of the main root (root cap: 0-5 mm, mid root: 10-15 mm, root base: 15-20 mm), fully expanded 3 leaf and main stem were hand-sliced into ≈80 µm thick cross-sections, and incubated in lactic acid saturated with chloral hydrate at 70°C for 1 h to clear the sections. For suberin determination, the sections were stained with Sudan red 7B (Brundett *etal*., 1991), air-dried for 20 minutes at room temperature, then observed using a bifocal microscope equipped with a camera. For lignin determination, the sections were stained for 5 minutes with phloroglucinol/hydrochloride (20 g phloroglucinol in 80 mL of 20% ethanol solution with 20 mL

of concentrated 12 N HCl added to it) (Jensen, 1962), air-dried at room temperature, and then observed using the same microscope as above.

Qualitative determination of root oxygen release: Radial Oxygen Loss

Rice genotypes contrasting for iron toxicity tolerance were grown in hydroponics under control nutrient solution (Famoso *et al*., 2010) for 14 days before subjecting them to excess Fe^{2+} (300ppm) treatment up to 28 days of growth. At day 10 and 14 of Fe^{2+} treatment, a single plant per genotype was carefully removed from the hydroponic trays and used to set the radial oxygen loss (ROL) experiment.

The intact root system of 24 and 28day-old plants (correspond to 10 and 14 days after Fe treatment) were immersed in transparent chambers (50/250 mm, width/height) containing agar-water medium (0.05% w/v) with0.012 g methylene blue per liter of solution and 0.13 g sodium dithionite. Addition of sodium dithionite reduces the medium into a colorless solution, as described in the reaction below. Throughout the preparation time, the solution was deoxygenated by flowing-in oxygen-free nitrogen gas until the plants are immersed in the chambers.

$$MeB^{+} + 2H_2O + S_2O_4^{-2} \longrightarrow MeBH + Cl^{-} + 3H^{+} + 2SO_3^{-2}$$

Blue Colorless

The shoot base of immersed plants was fixed into a rubber lid on the top of the chamber so that the roots were in an O_2 free medium and the shoot was in the air. The chambers with the plants were incubated at a constant temperature of 30°C for 48 hours. The development of blue color around the roots was observed every hour and pictures were taken using a camera. The intensity

and the speed at which the blue color developed were used to infer varietal differences in O_2 diffusion via the aerenchyma, from the shoots to the roots.

3.1.2.5 Statistical analysis

Statistical analysis of the data was done using the Statistical Tool for Agricultural Research (STAR) and R i386 version 3.2.2. Analysis of variance (ANOVA) was performed using the general linear model (GLM) procedure. Treatment means were separated using Duncan's multiple range test (DMRT) at $P \leq 0.05$. Correlation analysis was done to establish the relationship between pairs of variables. The percentage of aerenchyma was quantified using GIMP 2.8 (Media Cybernetics, Bethesda, MD, USA) and calculated on total tissue cross-sectional area.

3.2 Gene expression analysis using qPCR

The aim of this study was to analyze the transcriptional regulation of key genes involved in iron uptake and homeostasis in response to iron toxicity.

3.2.1 Experimental site, plant material, design and treatments

The experiment was conducted at AfricaRice center in Dar es Salaam, Tanzania using two varieties, CK801 (tolerant) and Supa (sensitive),selected based on the results obtained in Activity 1 and 2. In the screen house, the hydroponicexperiment using modified Magnavaca nutrient solution (Famoso *et al*., 2010) was set up in a complete block design and three reps. Two Fe treatments, control (2 ppm Fe^{2+}) and Fe-toxic (300 ppm Fe^{2+}) both supplied as $FeSO_4.7H_2O$, were applied.All plants were grown in the control nutrient solution for fourteen days, after which Fe treatment was imposed for another 14 days. The pH of the solution was adjusted daily to 4.2 using automatic pH meter by either adding NaOH if the pH is low or HCl if the pH is high. The

amounts of iron and phosphorus present in the Fe-toxic treatment were determined daily through Ferrozine and phosphorus assays, adding back into the solution as necessary to maintain the concentration throughout the experimental period.

3.2.2 Phenotyping of rice plants and tissue sampling

Measurementsof morphological traits (shoot height, root length, functional lateral root number, number of green and dry leaves and leaf length) were done every two days from the time Fe treatment was initiated. Root and shoot samples were collectedevery two days at midday starting from day 3 of the iron treatment.For each tissue (root and shoot) and sampling point, one composite sample per Fe treatment was obtained by pooling samples collected from nine plants (3 plants per treatment ×three replications). All tissue samples were flash-frozen in the liquid nitrogen and conserved at -80°Cprior to RNA extraction. At the end of theexperiment, remaining rice plants were harvested and their shoot and root fresh weights were determined separately. The tissues were then oven dried at 70°C for 3 days before measuring their dry weights. The dried tissues were finely ground and used for tissue Fe analysis.

3.2.3 RNA extraction and cDNA synthesis

Total RNA samples were extracted from finely ground rice roots and shoots using the Tri-reagent. To prevent Genomic DNA from contaminating the samples, the RNA extracts was treated with DNase I using Direct-zol RNA purification Kit (Zymo Research), according to the manufacturer's instructions. RNA quality was evaluated by determining absorbance ratio at 260 nm/280 nm (all samples should be within the range of 2.0 to 2.2) and by checking RNA integrity by electrophoresis on 1.2 % agarose gel. Complementary DNA synthesis was done using 0.5 micrograms of the total RNA using Revert Aid First Strand cDNA Synthesis Kit (Zymoresearh),

and primer oligo-dT in a total volume of 20µL. Primers of the target genes and reference gene, Ubiquitin,(Table 3)were designed using Primer-BLAST (NCBI).

3.2.4 Real time-qPCR

The synthesized cDNA was used as template in the real time-qPCR assays.The reaction was carried out in a volume of 25 µL containing 2 µL of 1:2 diluted cDNA, 0.3µM primers and 12.5µL Maxima SYBR Green/ROX qPCR Master Mix 2× (Thermo Fisher Scientific Inc.). PCR conditions for thermal cycling on BIO-RAD CFX 96 qPCR (BioRAD laboratories Inc.) were as follows: 95 °C 10 minutes, 40 cycles of 95 °C for 15 seconds, (51-62) °C for 30 seconds, 72 °C for 30 seconds and 51 - 95°C at 0.3°C per second. Fluorescence data were automatically recorded during each cycle at 72 °C.

3.2.5 Statistical analysis

Statistical analysis of all morphological traits was performed based on a Split plot randomized complete block design model with three replications using Statistical tool for Agricultural Research (STAR) (IRRI, 2013) and R i386 version 3.2.2. (The R Foundation for Statistical Computing Platform, 2015). Associations between parameters were examined using Pearson'scorrelation analysis. Treatment means were separated using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$. Gene expression analysis was done using BIO-RAD CFX Manager Software version 1.6 (Bio-Rad Laboratories Inc., 2009) based on the threshold cycle (ΔCt) of control samples and the Fe treated samples, Normalized relative expression was based on the ΔΔCt of the target gene and the reference gene. Amplification efficiency of all primers in this study was tested with serial dilutions of pooled cDNA templates and was always >90.

Table 3: List of genes and their sequences as generated from primer-BLAST

GENE NAME	MSU/TIGR IDs	Forward primer (5'- 3')	Reverse primer (5'- 3')
OsSAMS2	LOC_Os01g22010.1	GTCAAGCCGCTCAAGTATGA	ATGGAGGTAGATAGCGAGAGAG
OsNAS1	LOC_Os03g19427.1	TGGGACGTACAGTTGTGTTG	TTCCTCGTTTACACTGGCTTAC
OsNAAT1	LOC_Os02g20360.1	GAATCTATTGCGGACAAGAACAC	CCTCTGCCACCTTGGATAAA
OsDMAS1	LOC_Os03g13390.2	CTGCTCTGAGGAACGTAACAA	CGTGTGCACTCCGGAATTA
TOM1 (OsZIFL4)	LOC_Os11g04020.1	TGACACACTTGTCAGGGTTTAG	GAAGTGCCCGTTATGATAGTT
OsYSL15	LOC_Os02g43410.1	TTTGGACCTTCCCTTCTTCTATC	CACACTACACCTCTTAGCTTCC
OsFRO1	LOC_Os04g36720.1	GGCGATCCTGAGTGACATAATC	GAGAGCTCGTTGGACTTCTTC
OsFRO2	LOC_Os04g48930.1	GGGAATTGTGACAGGCATAGA	GAGAAGTCGAGGTTGATGAAGG
OsIRT1	LOC_Os03g46470	ATGTGCTTCCACCAGATGTT	GTCGTGGTGGAGAAGAAGAAG
OsNRAMP1	LOC_Os07g15460.1	AGTCAAGCCTCCGGTTAATG	CAGGGCAGAGTGCAAGAATA
OsYSL2	LOC_Os02g43370.1	GGATGGCCTTTACCACTTCATA	TGTATCCTCATCTGTCGCTTTC
OsYSL18	LOC_Os01g61390.1	TTGGGCTCTGCCAAGAATAG	AAGAGGAGAACGGAGGAAGA
OsFRDL1	LOC_Os03g11734.1	GCTGGCACACAGACGATAA	ACTAGGCAGGGTATGGAGATAG
PEZ2	LOC_Os03g37640.1	AGTGTGAGAGTGAGCAATGAG	CGCAAGGATGGCAGAGATTA
OsVIT2	LOC_Os09g23300.1	GTTTGAGTTGGGACTGGAGAA	CAAACATGTAGGGTAGGAGTGG
OsMIT	LOC_Os03g18550.1	CTGTGAGCGCTTCTCAAGTA	ATTCTTGGCTTCCATCCTCTC
OsMIR	LOC_Os12g18410.1	ACGACTAGCCATCGGATTTG	GGCACACTCCCAATTACATAGA
TIC21 (similar to PIC1)	LOC_Os02g09470.1	TTTGGGTACTCTTGGGTTCTG	GCTGTCACCTTCCCAGTAATAA
OsFer1	LOC_Os11g01530.1	GAATGGAGGAGACTGTGTGAAG	TTGCTACTGCGTGCTTAATTTG

OsFer2	LOC_Os12g01530.1	GGCAGTAGTAGGTTTCGTTTCT	TTGCTACTGCGTGCTTAATTTG

Table 3cont'. List of genes and their sequences as generated from primer-BLAST

GENE NAME	**MSU/TIGR IDs**	**Forward primer (5'- 3')**	**Reverse primer (5'- 3')**
OsIRO2	LOC_Os01g72370.1	CCTCCTACCCAGCTAACTACT	CTTGTCGCTAGAGAGAGAGAGA
OsIRO3	LOC_Os03g26210.1	GAGGCATCTTCTTGGCCTATT	CAGACCCTCTCAATCTCTACCT
OsbHLH133	LOC_Os12g32400.1	GCAGAGAGCTCACAGTCTTATT	TCCTCTGTCTCCTGTTCTGT
OsIDEF1	LOC_Os08g01090.1	TCCCATAGAAGGACCCATATCT	TGCTGTTTCTGGTTTCCAATTC
Glutathione transporter	LOC_Os06g03560.1	GAGAAGGAAGCACGGAGAAA	GAATAGGTAGCCTGGGAAGATG
GST	LOC_Os01g49710.1	CTACTTCGACAGCACGGTAATC	CACGGCGGGAAGAACTTAATA
GST	LOC_Os01g49720	CGAGCTGAACCTGAACACAA	CGTGTCAACGTACTCCTCAAAG
Peroxidase (OsPx4)a	LOC_Os10g02040.1	CGACACGACCGTATAGGAGTA	GCCAGAGAGGAAGAAGAAGAAG
Peroxidase (POX)	LOC_Os06g16350.1	GCTCTGATGCTTCGTCTTCA	TTCTGCCTTCTTCTCTCCAATC
OsCAT-A	LOC_Os02g02400.1	CGGATAGACAGGAGAGGTTCAT	ATCACACTGGGAGAGGTAGTT
OsCAT-B	LOC_Os06g51150.1	AGCCAAGTCCAAAGACCAATA	CATCGTCAAAGAGGAAGGAGAA
OsCAT-C	LOC_Os03g03910.1	CCCACGTACTCCACATCATT	CACACACCGCCCTACTTATAG
Fe-SOD (sodB)	LOC_Os06g05110.1	TGTCCTGCACGGTGAAATAG	CGATGGCCCATACTCTGTAATC
Mn-SOD	LOC_Os05g25850	GGTGTGGCTAGCTTTGGATAA	TGCTCCCAGACATCAATTCC
Cu/Zn-SOD	LOC_Os03g22810	GGTCTCCATGGGTTCCATATTC	TTTCCGGCAGGATTGTAGTG
sAPX (L-APX 7)	LOC_Os04g35520	ATCTCCTGGTCTTGCCTACA	CTTCTTGGTCCTCTGCGTATTT
tAPX (L-APX 8)	LOC_Os02g34810	ACTCGATGAAGCAGAAGATCAG	AGAGCATGATGTTGAGGAAGTAG

cAPX (OsAPXb, L-APX 2)	LOC_Os07g49400	CTGTCGTGGCGGTTGTAATA	GACAAAGAGCATCAAAGCAAGAA
AO	LOC_Os09g20090.1	AGTGAACATGATGCGGTGTAG	GACGTGAACTGGTAGCTGATG
GR	LOC_Os02g56850.1	GACCAGATGCACCAGAGATTAT	CAGACGGGTGAATACCAACA
Auxin efflux carrier Tansporter	LOC_Os01g45550.1	GCCGCTCCTGCATGTATAA	CATCTCCTCAGCCTATCTCAATC
Potassium channel AKT1	LOC_Os01g45990.1	CACCTCTCCTCTCTTCTCTTCT	GGGAATCTCTCGCCTTGAATAC
No apical meristem protein	LOC_Os01g48130.2	CTCCTCTCCTCCTTTCTCTCTC	AAAGCCTCTCTCTCTCTCTCTC
Beclin-1	LOC_Os01g48920.1	GTGGATTCCTTCGCCGATAA	GCTTGGATAGCACGACATAAGA
Methyltransferase	LOC_Os01g49250.1	GTTGTCACCCGAAGAACTAGAG	CCGTACAGAAGGGCTAAACTAC
UBQ5	LOC_Os01g22490.1	GAAGGAGGAGGAAATCGAACTG	CTTCACAGAGGTGATGCTAAGG

3.3 QTL mapping in two biparental populations

The aim of this study was to map QTLs for morphological and physiological traits related to tolerance to Fe toxicity.

3.3.1 Crosses and development of a mapping population

Based on the results obtained in the previous experiments,CK801 (donor parent) and Supa (recipient parent) were selected for the development of a mapping population. Since Supa is also a popular variety with good grain quality, breeding lines could be derived from this population and improved varieties developed. In 2014, crosses were initiated at Africa Rice Center, Morogoro, Tanzania. About 10 F_1 seeds were planted individually in pots. At three-leaf stage, small leaf samples were collected and genomic DNA was extracted following Romero *et al.* (2014). Microsatellite SSR markers that were polymorphic between the two parents were used to confirm heterozygous F1 plants. Two true F1 plants were selected and grown to maturity. F2 seeds were harvested and a set of 200 seeds was shipped to IRRI for further advancement using their rapid generation advancement (RGA) facility.

3.3.2 F2 genotyping

In 2015, F2 plants were grown at IRRI following the standard protocol of the RGA unit. After 14 days of growth, leaf discs were sampledand gDNA was extracted using a semi-automated oktopure method (oktopure, Biosearch technologies). The samples were genotyped with 4000 Single Nucleotide Polymorphisms (SNPs) using Infinum 4000.

3.3.3 F3 phenotyping

F3 seeds were obtained from 167 F2 plants out of the initial 200. Together with the two parents (CK801 and Supa), the F_3 progenies were grown hydroponically in a growth chamber under both control (0 ppm) and toxic Fe (300 ppm) conditions. The experiment was randomized using augmented design with each line repeated 4 times.

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Seeds were pre-germinated in the dark for 48 hours in petri dishes then transferred to the screen house in 40 L plastic tabs with grid fixed on the underside of a polystyrene plate perforated with 8 x 22 holes and floating on half strength modified Magnavaca nutrient solution (Famoso et al., 2010). After 7 days, the culture solution was adjusted to full strength, for another one week.Afterwards, excess Fe^{2+} was applied in the form of $FeSO_4.7H_2O$ added to the nutrient solution and plants were harvested after two weeks of excess Fe^{2+} treatment. The pH of the solutions was daily adjusted to 4.2 and the solution was replaced once a week. Ferrozine and phosphorus assays were done following section 3.1.1.3.3 and the condition of the hydroponic screening was the same as described in section 3.1.1.3.2.The tanks were daily rotated with each other to limit the effects of differences in environmental conditions.

Morphological and physiological traits including shoot length, leaf bronzing score, chlorophyll a, b, and carotenoids, number of green leaves, shoot and root fresh and dry weights were determined in both parents and the entire population using the same methodology and equipment as described in section 3.1.1.5. Based on the observations, 30 tolerant and 30 sensitive lines were selected at the end of the experiment for tissue Fe analysis. This was done only onplants grown under excess Fe, following the same protocol as in section 3.1.2.2.3.

3.3.4 QTL mapping

QTLs were mappedby composite interval mapping (Zeng 1994) using the software QTL CARTOGRAPHER version 1.17 (Basten et al., 1994; 2004). The window size used was 10 cM and the walking speed, 2 cM. A 1,000 permutation test was realized for each parameter in order to determine the threshold values to accept a QTL.Each marker was analyzed through single marker analysis (one-way ANOVA) to determine the markers potentially associated with iron toxicity tolerance. In addition, interval mapping was carried out using the Qgene software.

3.3.5 Statistical analysis of phenotypic traits

Analysis of variance (ANOVA), mean comparison between parents and among progenies for the traitsmeasured and simple correlation between markers were performedusing STAR version 2.1.1.

CHAPTER FOUR: RESULTS

4.1 Morphological and physiological responses of seventeen rice genotypes to iron toxicity

4.1.1 Variation of pH, iron and phosphorus in the culture solutions

At the beginning of the experiment, almost full amounts of ferrous was added back to the solution to maintain the concentrations at 300ppm. Phosphorus addition had a declining trend from day 1 of treatment to day 10. Ferrozine and phosphorus controls maintained almost the same trend throughout the experiment period (Fig.7).

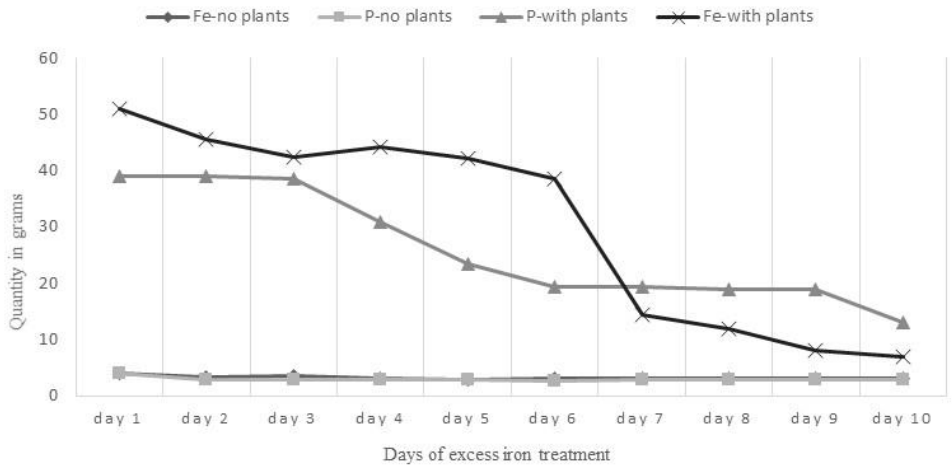


Fig 7:Daily amount of phosphorus (P) and iron (Fe) required to maintain the concentration of P and Fe2+ constant in the culture solution from the time excess iron was applied throughout the duration of the stress.

4.1.2 Morphological response in hydroponics solution

Time as a variable factor was highly significant ($p<0.001$) for all parameters measured apart from root length. Iron treatment as a fixed factor was not significant ($p>0.05$) for leaf width and root length. Strong to moderate significance ($p<0.01$) was recorded for variety with all the parameters except shoot and root weights, cellular CO_2 and conductance. Interactions between Fe treatment x time were significant ($p<0.05$) for all parameters measured apart from lateral roots, root length, cellular CO_2 and conductance. Variation of leaf bronzing and chlorophyll fluorescence was significant ($p<0.05$) for all the treatment and interactions (Table 4a).

Table 4a: Effects of variety (Var), iron treatment (Fe), stress duration (time) and their interactions on the variation of morphological and physiological traits measured

Traits	Var	Fe	Time	Fe x Var	Time x Var	Time x Fe	Time x Fe x Var
Leaf length	***	**	**	***	**	**	ns
Leaf width	***	ns	**	ns	ns	*	ns
Shoot length	***	**	**	*	*	*	ns
Root length	***	ns	*	ns	ns	ns	ns
No. green leaves	***	***	***	ns	ns	**	ns
No. Lateral roots	***	*	*	ns	ns	ns	ns
Shoot fresh weight	Ns	***	-	ns	-	-	-
Shoot dry weight	Ns	***	-	ns	-	-	-
Root fresh weight	*	***	-	ns	-	-	-
Root dry weight	Ns	***	-	ns	-	-	-
Leaf temperature	***	**	***	ns	ns	*	ns
Photosynthesis rate	**	***	***	ns	ns	***	ns
Cellular CO2	Ns	*	***	ns	ns	ns	ns
Transpiration rate	**	***	***	ns	ns	***	ns
Stomatal conductance	Ns	***	***	ns	ns	ns	ns
Chlorophyll content index	***	***	*	**	**	**	ns
Chlorophyll fluorescence	***	***	***	***	***	***	***
Chlorophyll a	**	*	-	*	-	-	-
Chlorophyll b	***	*	-	**	-	-	-
Carotenoids	**	*	-	*	-	-	-

**** P<0.001;**P<0.01; *P<0.05; ns= not significant; n= 306*

4.1.3 Effects of iron toxicity and stress duration on the parameters tested

After six days of toxic iron treatment, no significant difference ($p>0.05$) was recorded for most parameters apart from conductance, transpiration, Chlorophyll fluorescence, lateral roots, and leaf length. However, after twelve days of toxic iron treatment, all parameters had significant difference ($p<0.05$) across treatments with high response being recorded for control plants compared to plants subjected to iron toxicity except for leaf temperature and chlorophyll fluorescence that was high for iron treated plants than the control plants (Fig. 8).

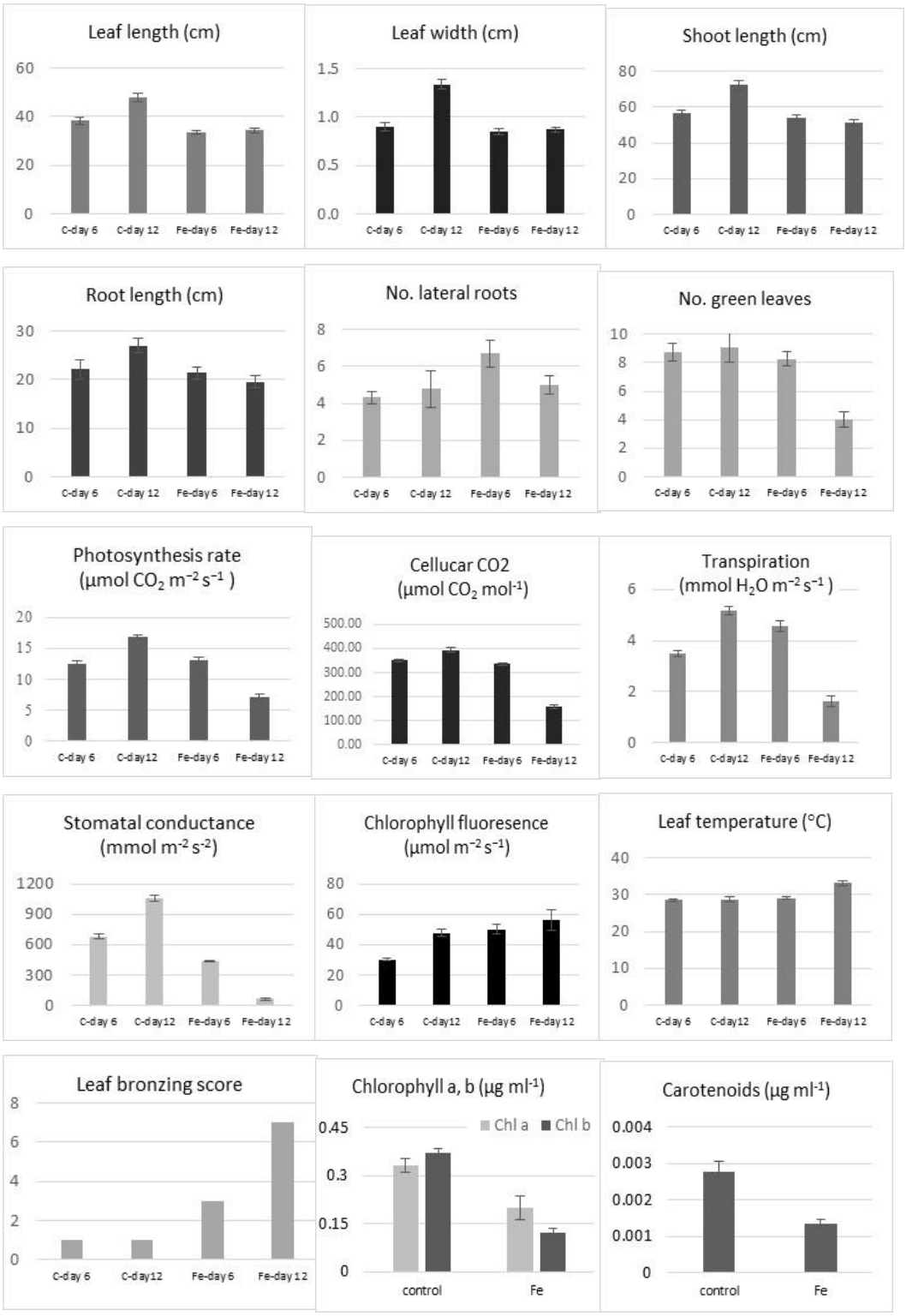


Fig 8:Mean variation of morpho-physiological traits based on iron treatment and stress duration.Error bars denote standard errors. "C" represent control and "Fe" represents excess iron conditions.

4.1.4 Varietal differences in leaf bronzing score over time

Leaf bronzing varied with time and variety during the toxic iron treatment. At day four of iron treatment, most varieties had a leaf bronzing score (LBS) of 1 (normal plant growth (IRRI SES, 2002) except 72-5, IR64, Azucena and Supa which showed a LBS of 3 and TOG 16771 with a LBS of 5. At day eight of toxic iron treatment, a clear variation between varieties was evident with few varieties including IR64,TOG6241 and AZUCENA scoring 9 (dead plant (IRRI SES,2002). At day twelve, only two varieties CK801 and Suakoko 8 had a LBS score of 3 while all the remaining 15 varieties had a LBS of 5-9 (Fig. 9).

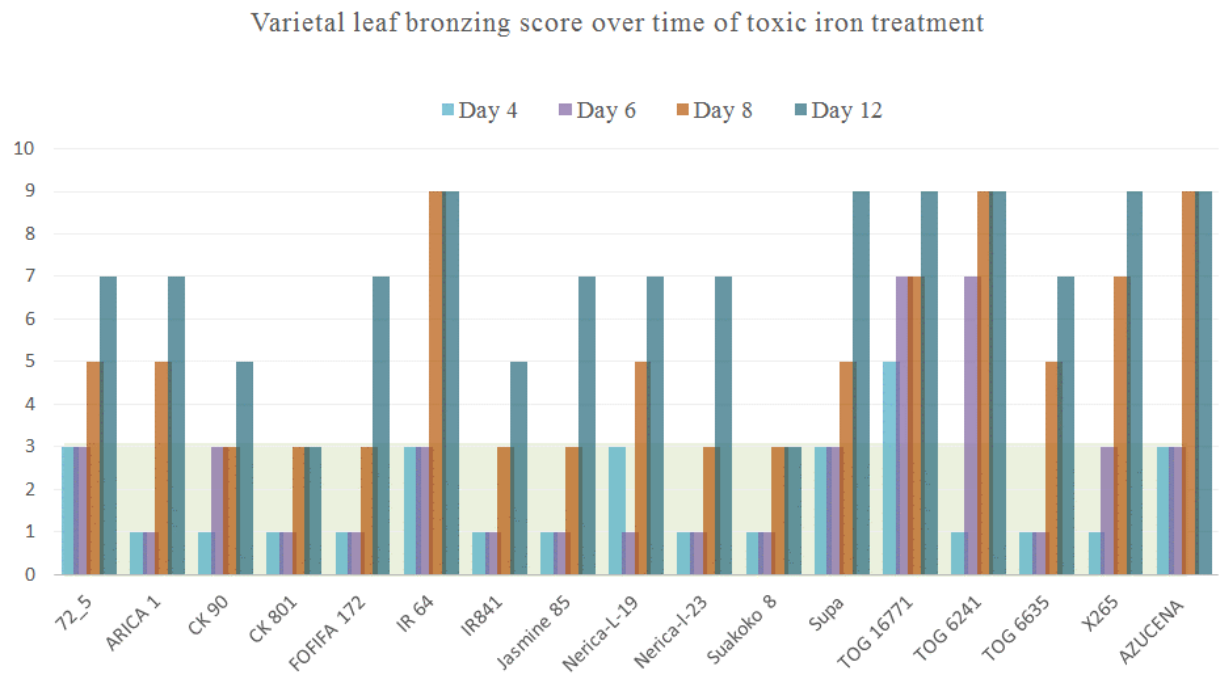


Fig 9: Leaf bronzing scores of the 17 genotypes tested under excess iron treatment.

4.1.5 Relationship between leaf bronzing and morpho- physiological traits

All physiological parameters were inversely related to leaf bronzing while morphological traits had a direct relationship with leaf bronzing. Number of green leaves (R=0.2956) indicated a strong linear positive relationship with leaf bronzing while at leaf bronzing seven the number of green leaves started reducing with increase in leaf bronzing. A weak negativelinear relationship was recorded between conductance (R=0.1631) and leaf bronzing while cellular CO_2 also exhibited a weak positive linear relationship with leaf bronzing (R=0.03) (Fig. 10).

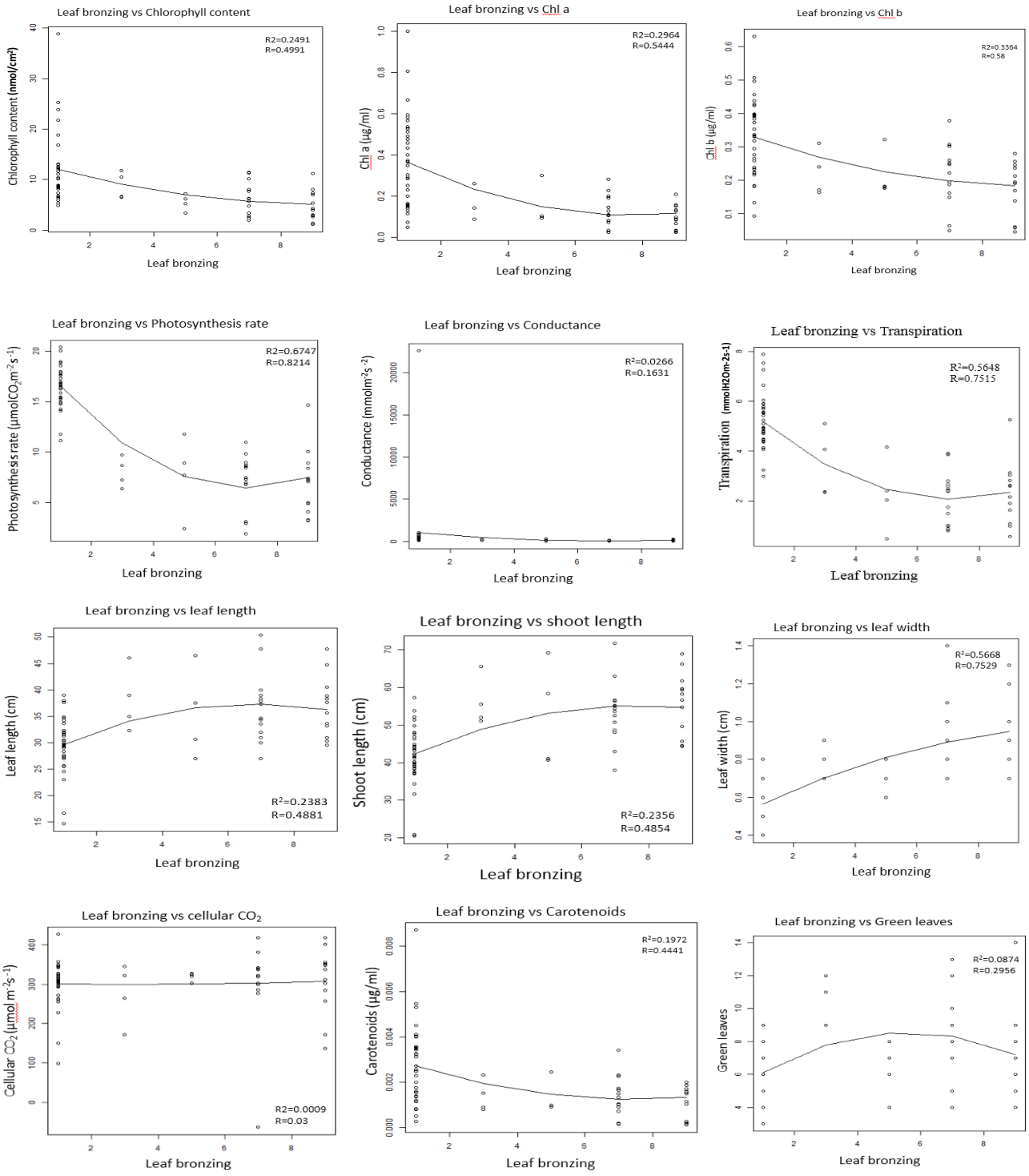
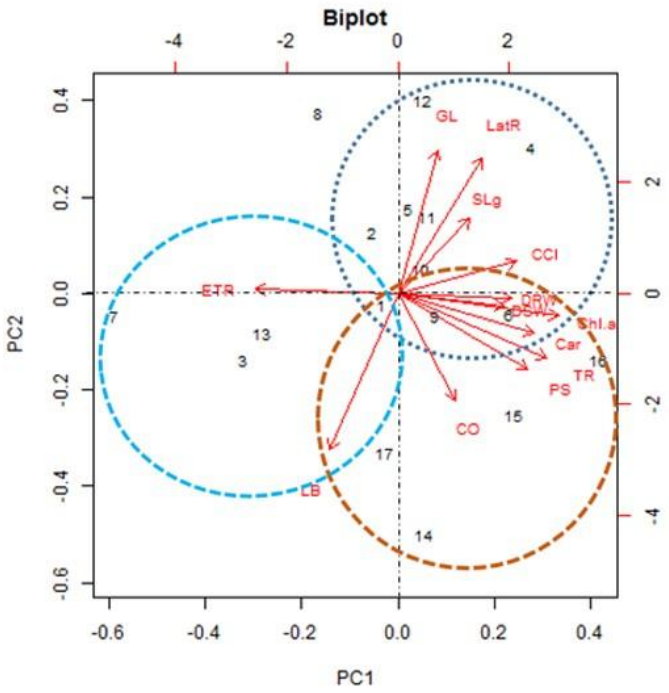


Fig 10: Relationship of morpho-physiological traits and leaf bronzing scores after 12 days of excess iron treatment.

4.1.6 Contribution of each trait to the variation observed and association with iron toxicity tolerance

A multivariate principal component analysis (PCA) and a biplot established the association between the traits measured and between the traits and the varieties. The PC1 and PC2 biplot established an inverse relationship between leaf bronzing and the number of green leaves, the number of lateral roots, shoot length, chlorophyll a content and photosynthesis rate, but a positive relationship with chlorophyll fluorescence (ETR). On the other hand, chlorophyll fluorescence was negatively related to all the other physiological and morphological traits analyzed. Using PC1 and PC2, varieties were clustered into three distinct groups. Suakoko 8, CK801, CK90, NERICA-L-23, ARICA 1, NERICA-L-19 grouped together with morphological traits including the number of green leaves, shoot length and number of lateral roots. IR64, Supa, and Azucena grouped together with leaf bronzing and chlorophyll fluorescence, whereas the third group consisting of TOG 6241, TOG 16771, X265, TOG 6635 grouped together with physiological traits including stomatal conductance, photosynthesis rate and transpiration. Out of the remaining four varieties three (Jasmine 85, FOFIFA 172 and 72-5) intercalated in between the three groups while one variety (IR841) did not lie in the range of any group (Fig. 11).



KEY	
Variety	**Code**
72_5	1
ARICA 1	2
Azucena	3
CK801	4
CK90	5
FOFIFA 172	6
IR64	7
IR841	8
Jasmine 85	9
NERICA-L-19	10
NERICAL-L-23	11
Suakoko 8	12
Supa	13
TOG 16771	14
TOG 6241	15
TOG 6635	16
X265	17

Fig 11: Multivariate principal component analysis.Varieties are coded from 1 to 17 as indicated in the table key. Traits were abbreviated as follows PS: Photosynthesis rate; ETR: Electron transfer rate; CO: Stomatal conductance; TR: Transpiration, CCI: Chlorophyll content index; Chla: Chlorophyll a content; Car: Carotenoid content; LatR: Lateral roots; SLg: Shoot length; GL: number of green leaves; DSW: Dry shoot weight; DRW: Dry root weight; LB: leaf bronzing

4.1.7 Varietal performance under stress and relationships among morphological and physiological traits

Interaction between variety and treatment elucidated intraspecific variation for various physiological and morphological traits measured. Under iron treatment, the variety TOG6635 recorded a high significant ($p<0.01$) response for chlorophyll content index, NERICA-L-23 high significant ($p<0.01$) response for Chlorophyll fluorescence, TOG 6241 and TOG 6635 high significant ($p<0.01$) response for leaf width, CK801 high significant ($p<0.01$) response for shoot length, leaf length and number of lateral roots, IR841 high significant ($p<0.01$) response for number of green leaves and FOFIFA 172 high significant ($p<0.01$) response for root length (Table 4b).

Table 4b:Variation of selected morphological and physiological traits among the varieties tested after 12 days of stress

	Leaf length (cm)		Leaf width (cm)		Shoot length (cm)		Root length (cm)		Number of green leaves		Number of lateral roots		LBS	Chlorophyll Content index		Chlorophyll fluorescence	
Variety	**Control**	**Fe**	**Control**	**Fe**	**Control**	**Fe**	**Control**	**Fe**	**Control**	**Fe**	**Control**	**Fe**	**Fe**	**Control**	**Fe**	**Control**	**Fe**
72-5	35.21^{c-e}	26.12^{fg}	1.05^{a-d}	0.6^{a-c}	51.29^{d}	36.62^{f}	22.12^{de}	25.93^{cd}	9.00^{c-e}	5.00^{e-g}	4.25^{cd}	6.75^{b-e}	7^{b}	15.1^{a-b}	3.8^{bc}	$45.5b^{c}$	40.50^{e}
ARICA 1	34.71^{c-e}	29.62^{c-f}	0.75^{e-g}	0.55^{a-c}	51.67^{d}	43.16^{b-f}	19.60^{ef}	17.88^{fg}	12.00^{ab}	7.75^{ab}	5.25^{b-d}	9.50^{a-d}	7^{b}	14.2^{a-c}	5.05^{a-c}	39.05^{bc}	81.75^{cd}
Azucena	38.00^{a-d}	28.83^{d-f}	0.95^{b-f}	0.65^{a-c}	52.75^{cd}	39.73^{ef}	23.82^{de}	25.35^{c-e}	6.00^{ef}	4.25^{fg}	2.50^{e}	5.50^{e}	9^{a}	14.05^{a-c}	3.35^{c}	49.05^{bc}	52.15^{e}
CK801	41.66^{a}	38.46^{a}	0.75^{e-g}	0.45^{bc}	62.29^{a}	55.33^{a}	20.50^{ef}	19.62^{fg}	8.00^{c-f}	6.25^{b-e}	6.00^{b}	11.25^{a}	3^{d}	16.0^{ab}	4.65^{a-c}	52.75^{bc}	47.85^{e}
CK90	39.79^{a-c}	35.71^{ab}	0.8^{d-g}	0.55^{a-c}	58.88^{a-c}	47.63^{bd}	22.60^{de}	22.12^{d-f}	10.50^{a-c}	7.00^{a-d}	5.00^{b-d}	9.25^{a-d}	5^{c}	13.75^{a-c}	3.95^{a-c}	42.0^{bc}	49.15^{e}
FOFIFA 172	33.92^{d-f}	28.50^{d-f}	0.85^{c-g}	$0.6a^{bc}$	54.21^{b-d}	41.09^{df}	33.22^{a}	35.17^{a}	6.75^{d-f}	5.25^{d-g}	4.25^{cd}	4.75^{e}	7^{b}	15.45^{a-c}	3.05^{c}	41.5^{bc}	59.35^{de}
IR64	29.33^{fg}	25.67^{fg}	0.7^{fg}	0.55^{a-c}	44.21^{e-g}	37.33^{ef}	21.23^{d-f}	20.25^{e-g}	8.25^{c-f}	4.00^{g}	4.50^{b-d}	10.00^{a-c}	9^{a}	12.6^{c}	5.0^{a-c}	32.4^{c}	183.35^{ab}
IR841	30.12^{e-g}	22.12^{gh}	0.65^{g}	0.4^{c}	42.88^{fg}	30.29^{g}	14.93^{g}	14.53^{g}	13.75^{a}	8.25^{a}	4.50^{b-d}	6.25^{de}	5^{c}	15.95^{a-c}	5.45^{a-c}	35.55^{bc}	180.7^{ab}
Jasmine 85	28.04^{g}	20.34^{h}	0.8^{d-g}	0.5^{a-c}	39.96^{g}	29.54^{g}	14.95^{g}	15.88^{g}	8.25^{c-f}	6.00^{b-f}	4.00^{d}	8.25^{a-e}	7^{b}	16.75^{a}	3.1^{c}	42.3^{bc}	196.6^{ab}
NERICA-L-19	41.96^{a}	34.50^{a-c}	0.85^{c-g}	0.55^{a-c}	61.54^{a}	48.38^{bc}	20.62^{ef}	19.18^{fg}	10.75^{a-c}	7.00^{a-d}	5.75^{bc}	6.50^{c-e}	7^{b}	13.75^{a-c}	4.2^{a-c}	36.075^{bc}	40.55^{e}
NERICA-L-23	34.16^{d-f}	26.08^{fg}	0.85^{c-g}	0.5^{a-c}	49.12^{d-f}	39.55^{ef}	13.35^{g}	15.05^{g}	9.50^{b-d}	6.00^{b-f}	4.75^{b-d}	6.75^{bc-e}	7^{b}	14.6^{a-c}	4.4^{a-c}	84.05^{a}	201.9^{a}
Suakoko 8	35.58^{b-d}	29.38^{d-f}	0.75^{e-g}	0.5^{a-c}	52.83^{cd}	41.86^{c-f}	23.38^{de}	18.43^{fg}	9.75^{b-d}	7.25^{a-c}	5.75^{bc}	9.50^{a-d}	3^{d}	12.95^{bc}	5.4^{a-c}	61.95^{ab}	51.9^{e}
Supa	41.50^{a}	33.00^{b-d}	1.0^{a-e}	0.4^{c}	62.29^{a}	47.38^{b-d}	17.18^{fg}	16.88^{fg}	5.75^{f}	4.25^{fg}	4.75^{b-d}	8.00^{a-e}	9^{a}	16.35a	4.1^{a-c}	36.68^{bc}	173.75^{b}
TOG 16771	33.71^{d-f}	27.12^{e-g}	1.1^{a-c}	0.7^{ab}	49.46^{de}	37.88^{ef}	28.68^{bc}	32.62^{ab}	8.00^{c-f}	5.50^{c-g}	4.25^{cd}	7.25^{b-e}	9^{a}	15.0^{a-c}	2.8^{c}	41.78^{bc}	86.25^{c}
TOG 6241	40.75^{ab}	29.21^{d-f}	1.15^{ab}	0.75^{a}	60.62^{ab}	42.71^{b-f}	30.90^{ab}	31.55^{ab}	8.75^{c-f}	6.75^{a-e}	4.25^{cd}	10.00^{a-c}	9^{a}	13.55^{a-c}	7.1^{ab}	56.2^{bc}	55.65^{e}
TOG 6635	42.04^{a}	31.38^{b-e}	1.25^{a}	0.75^{a}	59.79^{ab}	43.79^{b-e}	25.05^{cd}	29.02^{bc}	8.50^{c-f}	5.50^{c-g}	6.00^{b}	7.50^{b-e}	7^{b}	14.15^{a-c}	7.2^{a}	53.8^{bc}	198.75^{ab}
X265	35.79^{b-d}	32.46^{b-d}	0.75^{e-g}	0.55^{a-c}	58.54^{a-c}	49.19^{b}	19.75^{ef}	16.57^{fg}	9.00^{c-e}	6.50^{a-e}	7.50^{a}	10.25^{ab}	9^{a}	13.85^{a-c}	4.2^{a-c}	45.45^{bc}	56.0^{e}

Means with the same letter in the same column are not significantly different; n=306, means separated using DMRT

Most physiological traits indicated a strong linear correlation with the morphological parameters. Photosynthesis rate which was highly significant (P<0.001) with all the other parameters except for cellular CO2 and conductance had a strong positive linear correlation with transpiration (96%), shoot length (85%), number of green leaves (82%), leaf width (79%), leaf length (70%), number

of tillers (70%) and root length(62%). Consequently, a moderate to weak negative linear correlation was also recorded for photosynthesis rate and leaf temperature (69%) and fluorescence (26%). Cellular CO2 which was only significant (P<0.05) for fluorescence had a weak negative correlation with fluorescence (33%) (Table 4c).

Table 4c: Correlation matrix between physiological parameters across treatment

	Photosynthesis rate	Cellular CO2	Transpiration rate (mmol)	Flouresence (ETR)	Shoot length (cm)	Leaf length (cm)	No. green leaves	No. tillers	Leaf width (cm)	Root length (cm)	No. lateral roots	Leaf Temp.°C	Conductance
Photosynthesis rate	1	0.08	0.96	-0.26	0.85	0.7	0.82	0.7	0.79	0.62	0.1	-0.69	0.22
Cellular CO2	ns	1	0.16	-0.33	0.15	0.1	0.09	0.11	0.12	0.13	0.18	0.06	0.03
Transpiration rate (mmol)	****	ns	1	-0.31	0.85	0.68	0.82	0.73	0.77	0.6	0.09	-0.7	0.18
Flouresence (ETR)	****	**	*	1	-0.38	-0.27	-0.17	-0.23	-0.22	-0.31	0.04	0.25	-0.07
Shoot length (cm)	****	ns	****	**	1	0.88	0.75	0.63	0.78	0.52	0.19	-0.59	0.24
Leaf length (cm)	****	ns	****	*	****	1	0.66	0.56	0.71	0.41	0.26	-0.43	0.18
No. green leaves	****	ns	****	ns	****	****	1	0.83	0.71	0.43	0.25	-0.61	0.16
No. tillers	****	ns	****	ns	****	****	****	1	0.67	0.46	0.12	-0.53	0.17
Leaf width (cm)	****	ns	****	ns	****	****	****	****	1	0.66	0.28	-0.52	0.38
Root length (cm)	****	ns	****	*	****	***	****	****	****	1	0.1	-0.42	0.25
No. lateral roots	****	ns	ns	ns	ns	***	****	ns	****	ns	1	0.11	0.01
Leaf Temp °C	****	ns	****	*	****	***	****	****	****	***	ns	1	-0.18
Conductance	ns	ns	ns	ns	ns	ns	ns	ns	****	*	ns	ns	1

***** P<0.001;***P<0.01; *P<0.05; ns= not significant*

Transpiration rate which was significant (P<0.05) for fluorescence and highly significant (P<0.001) for all the other parameters except for lateral roots and conductance had a strong to moderate positive linear correlation with shoot length (85%), number of green leaves (82%), leaf width (77%),number of tillers (73%), leaf length (68%), root length (60% and a strong to weak negative linear correlation with leaf temperature (70%) and fluorescence (31%).Leaf temperature and fluorescence recorded a moderate to weak negative linear correlation with all the parameters while conductance which was only significant for leaf width (P<0.001) and root length (P<0.05) had very weak positive linear correlation with leaf width (38%) and weak negative linear correlation with fluorescence (7%) and leaf temperature (18%). Shoot length had a strong positive linear correlation with leaf length (88%), number of green leaves (75%), number of tillers (63%), leaf width (78%) and root length (52%). High significance (P<0.001) was also recorded for by shoot length for most parameters except for fluorescence which was significant at (P<0.01) and not significant for cellular CO2, conductance and number of lateral roots. Number of lateral roots did not record any appreciable correlation with any of the parameters but was highly significant P<0.001 for photosynthesis rate, number of green leaves, leaf width and very significant (P<0.001) for leaf length (Table 4c).

4.2 Morphological response in Geoponics solution

The interaction between growth media and genotype was significant and variation in morphological traits was observed in 6 weeks after initiating Fe^{2+} treatment as opposed to hydroponic experiment in which the same changes were visible in two weeks of growth.

Stress period, variety and treatment were significant ($p < 0.05$) for leaf length (table 10). Time x variety, time x treatment, variety x treatment were significant ($p < 0.05$) for leaf length.

However, treatment x variety x time was not significant ($p > 0.05$) for leaf length. Number of leaves was significant ($p < 0.05$) with time, variety and treatment. Interactions between time x variety and variety x treatment significantly ($p < 0.05$) increased number of leaves. Response of chlorophyll content was significant ($p < 0.05$) for time, variety, treatment, time x treatment, time x variety, and variety x treatment. (Table 5).

In geoponics; time, variety, treatment, time x variety and variety x treatment were found to be significant at ($p < 0.05$) for leaf length and leaf width while time x treatment significance ($p< 0.05$) was seen for leaf length only and not for leaf width. All factors and interactions; time, variety, treatment, time x variety, time x treatment and variety x treatment were very significant ($p < 0.001$) for number of tillers (Table 5).

Table 5: ANOVA table showing response of various parameters measured from the pot experiment

Source	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	No. tillers	No. leaves	Chlorophyll content SPAD	Leaf Temp °C
Time	****	****	****	****	****	****	****
Variety	****	****	****	****	****	****	****
Treatment	**	**	****	****	****	****	ns
Time: Variety	****	***	****	****	****	****	ns
Time: Treatment	****	****	ns	****	****	****	ns
Variety: Treatment	****	****	****	****	****	****	****
Time: Variety: Treatment	ns	ns	ns	Ns	ns	ns	ns
Mean	56.30	35.68	0.98	0.76	8.06	32.35	22.04
SE±	0.80	0.11	0.01	0.05	0.14	0.30	0.14

***** P<0.001, *** P<0.01, ** P<0.05, ns not significant*

All traits showed significance ($p < 0.05$) across time and treatment with peak performance indicated in week 10 for most traits apart from leaf length, number of leaves in Fe toxic treatment which had best performance in week 9 and chlorophyll in both treatment which had peak performance in week 9 however week 9 and week 10 was not significantly different ($p > 0.05$) (Table 6a).

Response of varietal traits to Fe toxicity varied significantly ($p < 0.05$) across the two treatments with variety CK90 recording the highest mean for plant height and leaf length in both treatments and NERICA-L-23 and IR841 recording the least means for the same traits in control and Fetox treatments respectively (Table 6b).

Table 6a: Trait performance across time and treatment in geoponic experiment

	Control treatment					Fetox treatment				
Time	**Plant height (cm)**	**Leaf length (cm)**	**No. leaves**	**No. tillers**	**Chlorophyll content**	**Plant height (cm)**	**Leaf length (cm)**	**No. leaves**	**No. tillers**	**Chlorophyll content**
Week 3	**24.21 g**	**20.35 f**	**3.46 g**	**0.00 d**	**23.28 d**	**23.40 f**	**19.81 e**	**3.44 e**	**0.00 c**	**24.13 c**
Week 4	36.01 f	**27.95 e**	4.90 f	0.04 d	25.54 cd	37.33 e	28.09 d	4.79 d	0.02 c	30.47 b
Week 5	48.99 e	35.43 d	6.71 e	1.25 c	26.78 c	47.38 d	34.18 c	6.38 c	1.27 b	36.55 a
Week 6	57.94 d	38.85 c	8.58 d	1.48 c	27.22 c	60.33 c	39.35 ab	9.21 a	1.60 b	38.12 a
Week 7	60.84 d	38.75 c	8.98 d	2.42 b	31.72 b	62.73 c	40.20 ab	8.50 ab	2.46 a	36.72 a
Week 8	70.51 c	39.39 c	10.00 c	3.25 a	32.08 b	67.53 b	40.08 ab	8.63 ab	2.25 a	36.07 a
Week 9	76.50 b	43.23 b	13.21 b	3.56 a	36.82 a	71.98 a	41.03 a	9.29 a	2.31 a	38.55 a
Week 10	80.85 a	46.19 a	14.67 a	3.69 a	36.67 a	74.26 a	38.02 b	8.19 b	2.52 a	36.88 a

Note: Means in a column followed with same letters are not significantly different at 95% level of probability, Mean separated by DMRT

Table 6b: Response of variety for different traits across treatment in geoponic experiment

	Control treatment						Fetox treatment					
Variety	**Plant height (cm)**	**Leaf length (cm)**	**Leaf width (cm)**	**No. leaves**	**No. tillers**	**Chlorophyll**	**Plant height (cm)**	**Leaf length (cm)**	**Leaf width (cm)**	**No. leaves**	**No. tillers**	**Chlorophyll**
ARICA 1	43.94 fg	30.91 fg	1.00 cdef	10.46 ab	2.25 abc	28.48 de	45.84 h	31.78 ef	0.99 bcde	10.38 a	2.46 a	35.96 abc
ARICA7	48.16 f	31.44 efg	0.88 f	11.13 a	2.42 a	31.20 bcd	47.54 gh	29.25 f	0.81 f	9.46 ab	2.08 ab	37.30 ab
ARICA8	56.42 e	35.55 cdef	1.08 bcd	10.08 abc	2.29 abc	31.10 bcd	53.65 efg	36.58 cde	0.97 bcde	8.29 bcde	1.75 bcde	36.74 abc
CK801	64.90 bcd	43.58 ab	1.00 cdef	9.21 bcde	2.38 ab	32.42 bcd	60.99 abcd	41.15 abc	0.92 def	7.46 cdefg	1.63 bcdef	36.51 abc
CK90	73.95 a	47.37 a	0.94 def	8.42 cdef	1.92 abcde	30.44 bcd	67.68 a	45.25 a	0.8958 def	7.38 cdefg	1.88 abc	34.47 abc
FOFIFA172	64.58 bcd	34.74 def	1.08 bcd	7.96 ef	1.88 abcde	37.98 a	64.10 abc	31.97 ef	0.96 cdef	5.50 h	1.21 ef	39.09 a
IR 841	39.04 g	26.42 g	0.71 g	8.92 bcde	2.00 abcd	24.50 e	25.21 i	17.27 g	0.49 g	5.38 h	1.17 ef	19.46 d
Jasmine 85	55.57 e	36.56 cde	1.12 bc	9.75 abcd	2.21 abc	35.28 abc	48.53 gh	32.69 def	1.01 bcde	7.79 bcdef	1.58 bcdef	38.31 a
NERICA-L-19	56.69 e	38.61 bcd	0.92 ef	8.75 bcde	1.75 cde	28.77 de	59.14 bcde	35.9750 cde	0.88 ef	6.46 fgh	1.25 def	34.43 abc
NERICA-L-23	28.95 h	19.64 h	0.57 g	5.21 g	1.00 f	18.60 f	51.93 fgh	31.87 ef	0.89 def	6.96 defgh	1.38 cdef	37.80 ab
Suakoko 8	56.30 e	38.62 bcd	0.91 ef	9.75 abcd	2.17 abc	28.40 de	57.73 cdef	38.90 bc	0.89 def	8.96 abc	2.08 ab	33.00 bc
Supa	66.13 bcd	43.63 ab	1.03 cdef	8.33 def	1.79 bcde	30.78 bcd	65.92 ab	42.74 ab	1.04 bcd	6.38 fgh	1.13 f	36.14 abc
TOG 16771	59.43 de	33.22 ef	1.20 b	6.71 fg	1.50 def	30.24 cd	59.14 bcde	32.57 def	1.10 bc	5.46 h	1.17 ef	31.87 c
TOG6635	66.88 bc	40.24 bc	1.41 a	7.54 ef	1.38 ef	28.63 de	60.70 bcd	39.30 bc	1.35a	5.92 gh	1.04 f	32.79 bc
V1	60.22 cde	39.18 bcd	1.15 bc	10.42 ab	2.29 abc	28.00 de	56.96 def	36.68 cde	1.13 b	8.42 bcd	1.83 bcd	31.99 c
Yun Yine	70.59 ab	40.60 bc	1.04 cde	8.38 cdef	2.17 abc	35.36 ab	64.84 ab	37.52 bcd	0.95 cdef	6.67 efgh	1.25 def	39.07 a
Mean	**56.98**	**36.27**	**1**	**8.81**	**1.96**	**30.01**	**55.62**	**35.09**	**0.95**	**7.3**	**1.56**	**34.68**
CV%	**41.37**	**32.88**	**35.77**	**50.06**	**81.67**	**28.21**	**36.86**	**29.8**	**33.06**	**41.12**	**80.03**	**21.74**
SE±	**1.2**	**0.61**	**0.02**	**0.23**	**0.08**	**0.43**	**1.05**	**0.53**	**0.02**	**0.15**	**0.06**	**0.38**

Note: Means in a column followed with same letters are not significantly different at 95% level of probability, Means separated by DMRT

TOG6635 gave the best result for leaf width in both treatments with IR841 showing low performance in both treatments for leaf width. ARICA7 recorded the best performance for number of leaves and tillers in control treatment and ARICA 1 the best results in Fetox treatment for number of leaves and tillers. NERICA-L-23 recorded the lowest performance for number of leaves and tillers in control treatment while in Fetox treatment IR841 and FOFIFA172 had lowest recording for these traits. In control treatment FOFIFA172 recorded the highest chlorophyll content and NERICA-L-23 the lowest chlorophyll content. In Fetox treatment YunYine, Jasmine85 and FOFIFA172 recorded the best performance for chlorophyll content with IR841 recording the least chlorophyll content (Table 6b).

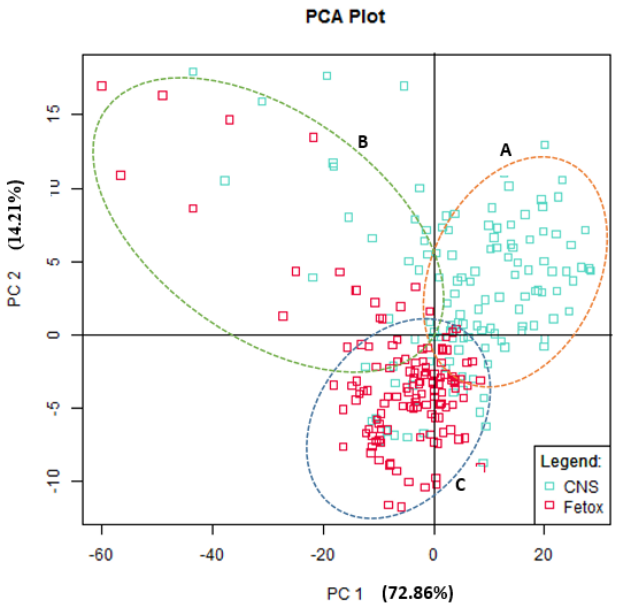


Fig 12: Principal Component analysis of phenotypic traits across treatment in geoponic experiment

Component (PC) analysis revealed that PC 1 (Plant height) and PC 2 (Number of leaves) could explain 87.07% of the phenotypic variance among varieties across the treatment. The analysis

revealed three distinct clusters: A (Phenotypic response due to Fetox treatment), B: (Similar phenotypic response across control and Fetox treatment), C: (Phenotypic response due to control treatment) (Fig. 12).Using Eigen values only PC1 and PC2 were seen to better explain the data as they could explain the correlation between the measured parameters up to 1.

4.2 Morphological and physiological analysis of four varieties contrasting for fe toxicity tolerance

Effects of treatments on the variation of the plant traits measured

For all the morphological, physiological and biochemical traits studied, the effect of Fe treatment was significant ($P \leq 0.05$) to highly significant ($P \leq 0.001$) on the variation observed. The effect of time was significant ($P \leq 0.05$) to highly significant ($P \leq 0.001$) for all the traits except transpiration, while variety effect was not significant ($P \geq 0.05$) for cellular CO_2 and conductance (Table 7).

For morphological traits, the interaction between Fe treatment and time (Fe x time), time and variety (time x Var), Fe treatment and variety (Fe x Var) and between Fe treatment, time and variety (Fe x time x Var) was significant ($P \leq 0.05$) to highly significant ($P \leq 0.001$) for all the traits measured. For physiological traits, the effect of Fe x time interaction was significant ($P \leq 0.05$ to $P \leq 0.01$) on the variation of all the traits while the effect of time x Var interaction was only significant at $P \leq 0.05$ and not significant for cellular CO_2 and conductance. The effect of Fe x Var interaction was significant ($P \leq 0.05$) to highly significant ($P \leq 0.001$) for all the traits except conductance. The interaction Fe x time x Var had a significant effect ($P \leq 0.05$ to $P \leq 0.01$) on trait variation except for transpiration and conductance. For the biochemical traits, all interactions had a highly significant effect ($P \leq 0.001$) on trait variations (Table 7).

Over the growth period in this experiment (3 weeks), overall performances of plants subjected to excess Fe were reduced compared with the control plants, except for chlorophyll fluorescence, lateral roots and starch content. Reduction of growth traits varied between 11.1% (root length) and 15.4% (shoot length) while metabolic traits reduced by 16.5% (soluble sugars) and 60% (proteins). The highest reduction among physiological traits was observed for conductance (61.9%), transpiration (53%) and photosynthesis (50.2%) (Table 7).

Table 7: Effects of iron treatment (Fe), variety (Var), growth stage (time) and their interactions on the variation of relevant plant traits measured in this study and average performance of plants grown under control and excess iron conditions over the duration of the growth period (3 weeks)

	Iron treatment	Time	Variety	Fe x Time	Time x Var	Fe x Var	Fe x Time x Var	Control means	Stress means
Morphological traits									
Shoot length	**	***	****	***	***	****	****	50.32	49.2
Root length	**	**	***	*	**	****	****	23.9	20.6
No. of lateral roots	***	***	**	***	**	***	***	4.3	6.9
Physiological traits									
Photosynthesis	**	*	*	*	*	**	**	17.5	8
Cellular CO_2	*	**	ns	*	ns	*	*	339.4	299.4
Transpiration	**	ns	**	**	*	*	Ns	5.3	2.5
Conductance	***	***	ns	**	ns	ns	Ns	543.7	206.9
Fluorescence	**	**	*	**	*	*	**	494.8	960.3
Relative water content	**	na	**	na	na	**	Na	81.6	58.7
Cell membrane stability	**	na	**	na	na	**	Na	86.7	74.1
Biochemical traits									
Soluble sugars	***	***	***	***	***	***	***	62.2	51.9
Proteins	***	***	***	***	***	***	***	0.3	0.1
Starch	***	***	***	***	***	***	***	18.7	21

*** P≤0.001; ** P≤0.01; * P≤0.05; ns= not significant; na= not applicable

Means in a column followed by the same letters are not significantly different at 95% level of probability

4.2.1 Morphological and physiological responses of the varieties depending on Fe treatment and stress duration

After 6 and 12 days of exposure to excess Fe stress levels, CK801 and IR64 maintained more or less the same shoot length in the stressed plants as in the control plants. After 6 days of stress, Suakoko8 boosted slightly plant growth compared to control plants but under severe stress, a slight decrease was noted. Shoot length in Supa decreased under both after 6 and 12 days of stress period (Fig 13). Similarly, CK801, IR64 and Suakoko8 showed minor difference of root length between stressed and control plants at 6 days of stress while in Supa, a decrease of root length was noted in stressed plants. After 12 days of stress period, decrease of root length was observed in all the varieties under excess Fe conditions. The number of new lateral roots, under both 6 and 12 days of stress period, increased in stressed plants of all the varieties compared with control plants. The largest increase of the number of lateral roots and the highest number under excess Fe conditions were observed in CK801 followed by Suakoko8, then IR64 and Supa (Fig. 13).

Concerning physiological traits, a reduction of photosynthesis rate was observed in all the varieties under both moderate and severe stress conditions with the decrease being much more pronounced after 12 days of exposure to excess Fe than after 6 days (Fig 13). The strongest reduction and the lowest photosynthesis rate under excess Fe conditions was observed in IR64. Under severe stress conditions, CK801 showed the highest photosynthesis rate among the four varieties. For cellular CO_2, there was no difference between stressed and control plants of IR64 and Suakoko8 at moderate stress level. In the same conditions, a slight increase of cellular CO_2 was observed in CK801 and Supa. At severe stress level, a decrease of cellular CO_2 was observed in all the varieties with CK801 showing the smallest variation between stressed and

control plants (Fig 13). Chlorophyll fluorescence increased under both moderate and severe stress levels in all the varieties with the values under severe stress being higher than in moderate stress. Under excess Fe conditions, CK801 presented the lowest chlorophyll fluorescence while Supa showed the highest (Fig 13).

Although the interaction between Fe x time x Var was not significant for transpiration and conductance, differential varieties' responses were also observed for these traits. At moderate stress level, transpiration significantly decreased in stressed plants of CK801 and IR64 compared to control plants while in Suakoko8 and Supa higher transpiration rate was observed in stressed plants. Transpiration rate decreased in all varieties under severe stress level. Stomatal conductance decreased at both moderate and severe stress levels in all the varieties but with different magnitudes (Fig 13).

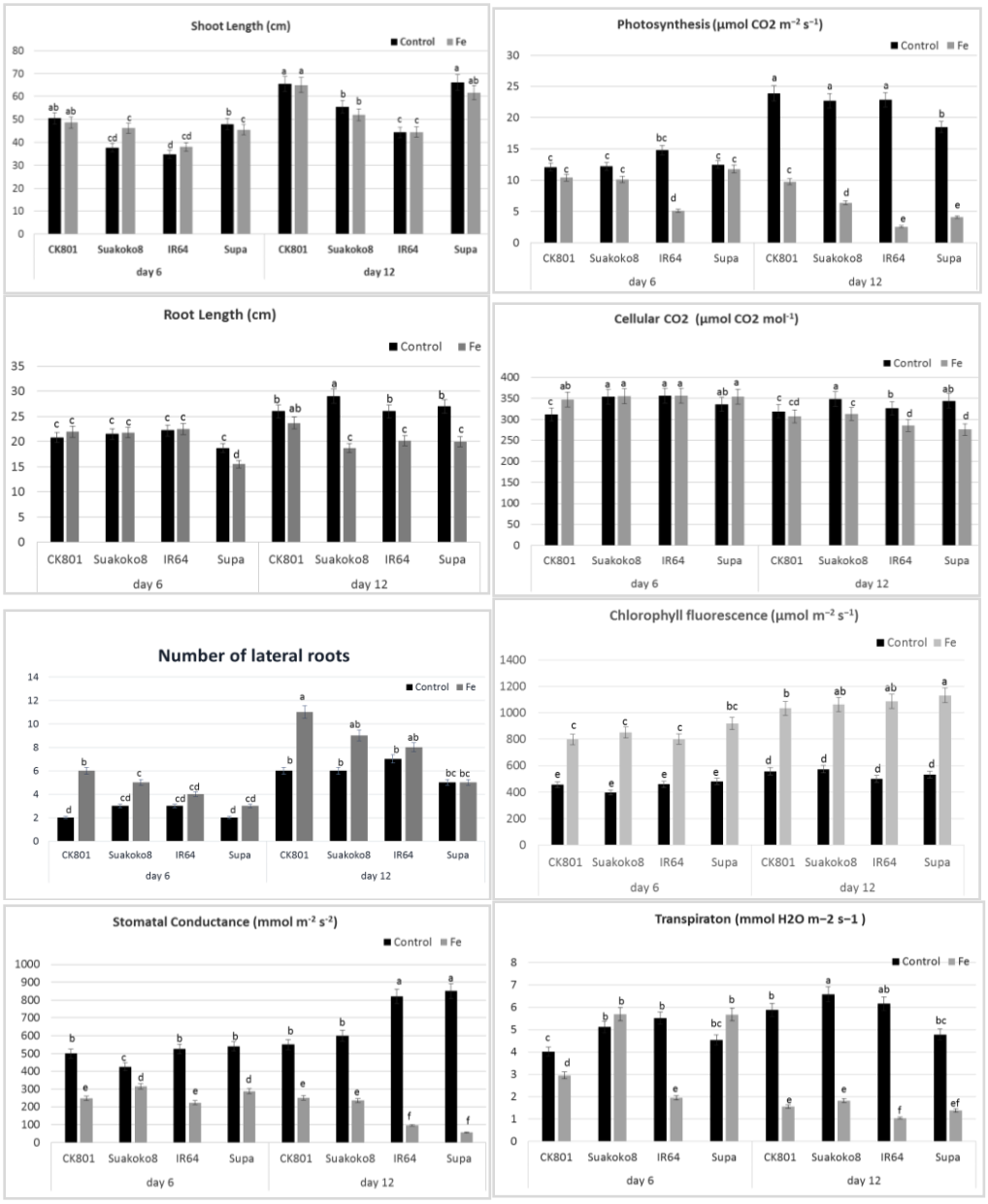


Fig 13: Differential morphological and physiological responses of the four varieties studied depending on the iron treament (control and excess Fe) and stress duration (after 6 days and 12 days of stress).

4.2.2 Tissue integrity and stress indicators

4.2.2.1 Chlorophyll, carotenoids, relative water content, and cell membrane stability

CK801 presented the robust plants under excess Fe conditions with an overall leaf bronzing score (LBS) of 3 and higher relative water content and chlorophyll content than the other varieties (Table 8). It was followed by Suakoko8 which recorded similar level of membrane stability than CK801 despite a higher LBS. IR64 and Supa presented comparable levels of leaf bronzing and chlorophyll content but IR64 had higher water content (within the same range as Suakoko8) than Supa. Suakoko8 recorded the highest carotenoid content followed by Supa andCK801, and lastly IR64 (Table 8).

For all the varieties, tissue Fe concentrations in the upper leaves were higher than in the lower leaves and in the roots (Table 8). Across tissues, the highest Fe concentration was recorded in Supa, followed by CK801 and IR64. Suakoko8 presented the lowest upper-leaf and root Fe concentrations among the varieties. Supa recorded the highest tissue Fe concentrations in the lower leaves and the upper leaves. The highest inner root Fe was recorded by CK801, followed by Supa, IR64 and Suakoko8 (Table 8).

Table 8: Varietal differences on plant traits measured only at the end of the experiment (relative water content, membrane stability, content of photosynthetic pigments) and only on stressed plants (leaf bronzing and iron concentration)

	RWC (%)		MS (%)		Chla (µg ml-1)		Chlb (µg ml-1)		Car (µg ml-1)		LBS	Root (µmol g-1)		LowLeaf (µmol g-1)	UpLeaf (µmol g-1)	Shoot (µmol g-1)
Treatment	Fe	Control	Fe	Control	Fe	Control	Fe	Control	Fe	Control	Fe	Fe	Control	Fe	Fe	Control
CK801	81.4^{a}	87.8 ^{a}	85.4^{a}	88.7^{a}	41^{a}	45.4^{a}	32.3^{a}	42.4^{a}	8.5^{ab}	7.9^{b}	3	1.35^{a}	0.04^{b}	0.38^{bc}	3.88^{b}	0.02^{b}
Suakoko 8	58.2^{b}	85.1^{a}	85.7^{a}	87.4^{a}	34.4^{b}	43.9^{a}	18.7^{b}	46.1^{a}	9.7^{a}	4.1^{d}	5	0.42^{c}	0.05^{a}	0.42^{b}	1.1^{c}	0.03^{a}
IR64	56.2^{b}	87.6^{a}	59.2^{b}	86.1^{a}	20.7^{c}	39.9^{b}	10.3^{c}	26.1^{b}	5.8^{c}	9.4^{a}	7	0.61^{bc}	0.04^{b}	0.41^{b}	3.33^{b}	0.01^{c}
Supa	38.3^{c}	85.8 ^{a}	66.2^{b}	84.5^{a}	19.9^{c}	43.9^{a}	9.6^{c}	41.9^{a}	8.2^{ab}	6.1^{c}	7	0.9^{b}	0.03^{c}	0.59^{a}	8^{a}	0.02^{b}

RWC: relative water content; MS: membrane stability; Chla: chlorophyll a; Chlb: chlorophyll b; Car: carotenoids; LBS: leaf bronzing score; Root: iron *concentration in root tissues of excess Fe treated and control plants; LowLeaf Fe: iron concentration in lower leaves; UpLeaf Fe: iron concentration in upper leaves:* shoot: *iron concentration in shoot of control plant. Means separated by DMRT*

4.2.3 Biochemical analyses

4.2.3.1 Changes in biochemical compounds in genotype tissue across treatment

Soluble sugar content in both roots and shoots was lower for the Fe-toxicity treatment compared with that for the control. However, the content of soluble sugars was higher in the shoots than in the roots. Across genotypes, Suakoko 8 recorded high soluble sugar in the control treatment compared with tolerant CK801 and sensitive IR64 and Supa. Under Fe toxicity treatment, Supa recorded the highest content of soluble sugars in contrast to tolerant Suakoko 8 and CK801 (Fig.14a).

There was higher starch content in the Fe-toxicity treatment compared with the control in both roots and shoots. As in soluble sugars, the amount of starch was higher in the shoots than in the roots. The genotypes exhibited the same high starch content in the Fe toxicity treatment, apart from IR64 whose starch content was high under the control treatment. IR64 recorded the highest starch content under both Fe toxicity and control treatments across all four genotypes. The lowest starch content was noted in CK801 under both Fe toxicity and control treatments (Fig.10a).The levels of MDA, which is a product of membrane lipid peroxidation, were observed to be higher in the Fe-toxicity treatment than in the control. However, equal levels of MDA were recorded in the roots and shoots in both treatments. CK801 recorded the lowest MDA level under Fe toxicity, followed by Suakoko 8. The highest MDA content was seen in Supa (Fig.14b).

Variation in TPC among the tissues was noted between experiments. Reduction of TPC in roots under Fe toxic conditions with a concomitant increase in the shoots under the same environment were evident.

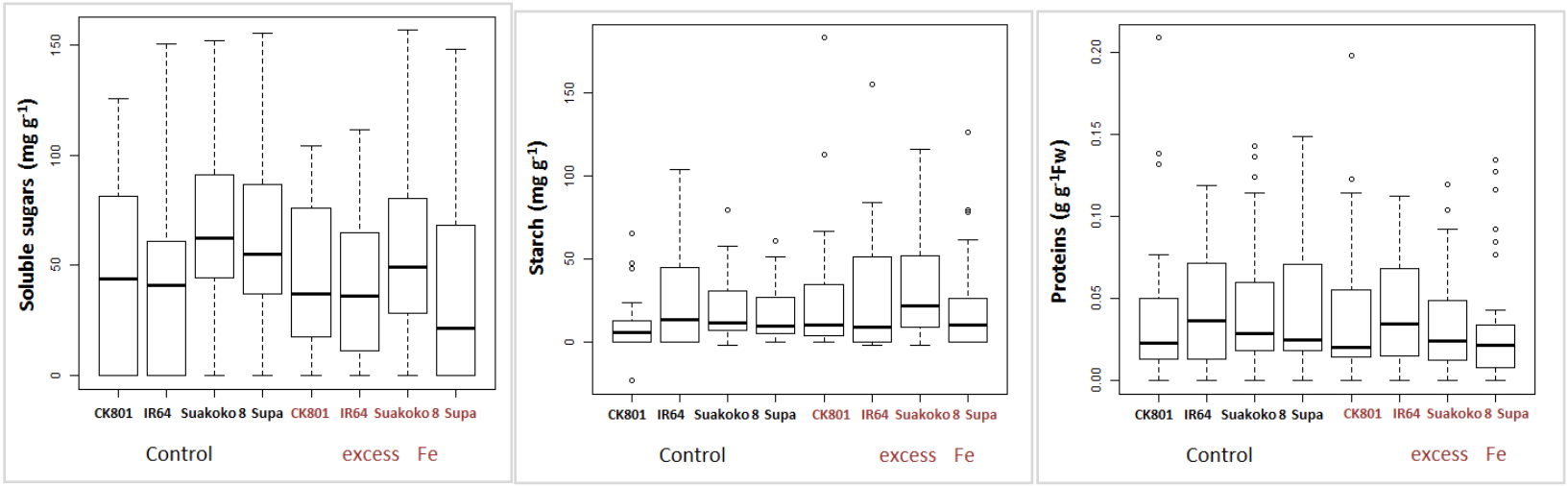


Fig 14a: Variation of biochemical traits (soluble sugars, starch and proteins) in four varieties contrasting for iron toxicity tolerance grown under control and excess iron (Fe) conditions

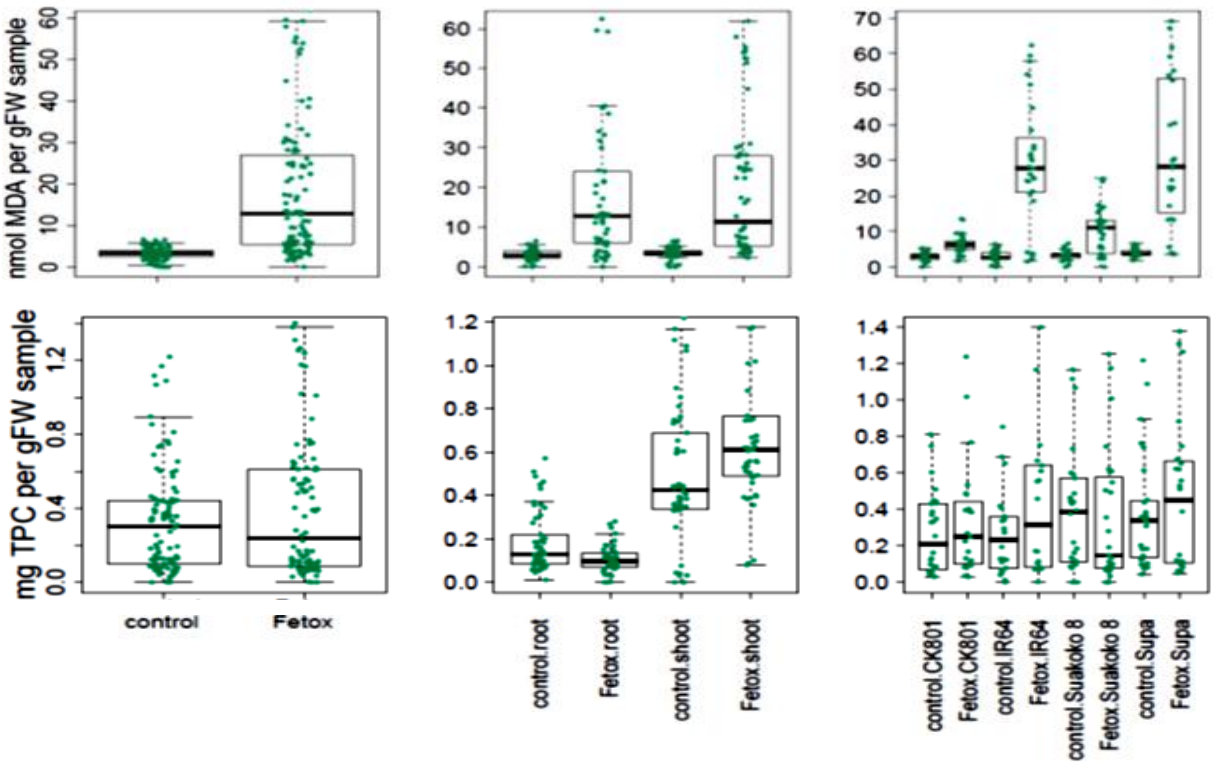


Fig14b: Soluble sugar, starch, MDA and TPC accumulation in the roots and shoots across treatments.

The sensitive genotypes in this study responded positively by accumulating higher levels of TPC compared with the tolerant genotypes across treatments. Suakoko 8 recorded very low TPC under Fe-toxicity compared with all the other genotypes (Fig.14b).

A correlation matrix between the traits elucidated a highly significant ($p<0.001$) correlation between soluble sugars and starch, soluble sugars and protein, soluble sugars and total phenolic compound; starch and protein, starch and total phenolic compounds; total phenolic compound and superoxide dismutase activity; ascorbate peroxidase activity and glutathione reductase activity. Soluble sugars was significant ($p<0.05$) to superoxide dismutase activity; starch and

peroxidase activity; protein and total phenolic compounds, peroxidase activity, superoxide dismutase activity; total phenolic compounds and peroxidase activity; catalase activity and glutathione reductase activity, peroxidase activity. No significant correlation was observed between MDA and the other traits (Table 9).

A strong (75%) positive linear correlation was observed between soluble sugars and starch. Positive (50%) correlation was observed between soluble sugars, and proteins, total phenolic, superoxide dismutase activity. Starch also showed positive correlation (50%) with protein, total phenolic compounds, superoxide dismutase activity. Protein also exhibited the same positive correlation with total phenolic compounds and superoxide activity. All the remaining interactions indicated a negative strong (75-100%) linear correlation (Table 9).

Table 9: Correlation matrix of biochemical parameters

	Total soluble sugar	mgstarch gDW	Total protein gFW	nMOL MDA. gFW	mg TPC gFW	APX Activity	CAT Activity	GR Activity	POX Activity	SOD Activity
Totalsoluble sugar	1.00									
mgstarch gDW	0.55****	1.00								
Total proteingFW	0.29****	0.23***	1.00							
nMOL MDA gFW	0.05 ns	0.06 ns	0.10 ns	1.00						
mg TPC gFW	0.35****	0.38****	0.17**	-0.06 ns	1.00					
APX Activity	0.04 ns	-0.03 ns	0.04 ns	-0.09 ns	0.01 ns	1.00				
CAT Activity	0.03 ns	0.10 ns	0.10 ns	-0.06 ns	0.09 ns	0.02 ns	1.00			
GR Activity	0.05 ns	-0.02 ns	0.11 ns	0.09 ns	-0.08 ns	0.3****	0.13*	1.00		
POX Activity	0.22***	0.17**	0.11*	-0.02 ns	0.14*	0.05 ns	0.13*	0.46****	1.00	
SOD Activity	0.14*	0.13 ns	0.17*	-0.02 ns	0.31****	0.18**	0.12 ns	0.02 ns	-0.06 ns	1.00

****P<0.0001, ***P<0.001 **P<0.01 *P<0.05 ns=not significant.

gDw=gram dry weight, gFw=gram fresh weight, APX=ascorbate peroxidase, CAT=catalase, GR=glutathione reductase, POX=peroxidase, SOD=superoxide dismutase, TPC=total phenolic compounds, MDA=malondialdehyde

4.2.3.2 Changes in biochemical parameters in genotype tissue over time

Soluble sugars

All genotypes displayed different trends for soluble sugar accumulation across sampling points and tissue types. For the shoot, CK801 and IR64 showed alternating trend in soluble sugar content accumulation with respect to Suakoko 8 and Supa. At sampling point 1, CK801 and IR64 had relatively low levels of soluble sugars, which dropped drastically at sampling point 2 but thereafter increased sharply at sampling point 3 for CK801 and at sampling point 4 for IR64. After sampling point 4, a slight increase and decrease were recorded for CK801 and IR64, respectively. Suakoko 8 and Supa recorded relatively high soluble sugar content at sampling point 1, which increased sharply at sampling point 2 before dropping drastically at sampling point 3. After sampling point 3, Suakoko 8 recorded a steady increase at sampling points 4 and 5, while Supa showed a slight decrease and increase at sampling points 4 and 5, respectively (Figure 14c).

For the root, each genotype had a distinct amount of soluble sugar at sampling point 1. Suakoko 8 and CK801 recorded an increase in soluble sugar content at sampling point 2, while Supa and IR64 showed a decrease at the same sampling point. At sampling point 3, CK801 and IR64 had an exponential increase in soluble sugar content; it then decreased at sampling points 4 and 5. On the other hand, Suakoko 8 revealed a sharp decrease at sampling point 3 and a steady decrease through sampling points 4 and 5. Supa recorded a slight increase at sampling point 3 and a slight decrease at sampling point 4 and 5.Overally Suakoko 8 had the highest amount of soluble sugars at sampling point 2 in both tissues(Figure 14c).

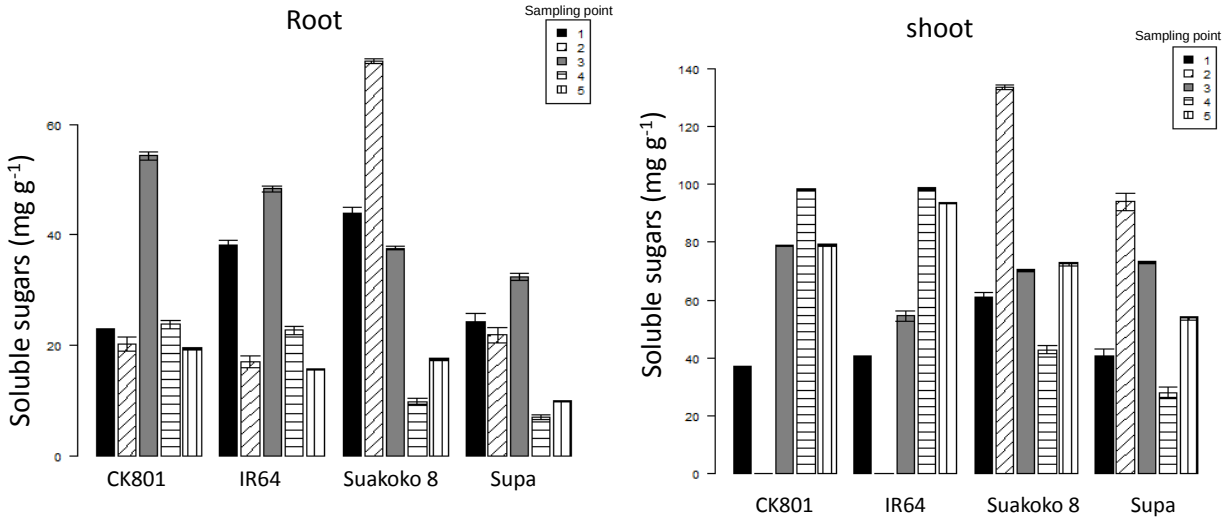


Fig 14c: Relative soluble sugar accumulation as per genotype root and shoot sampling points. Error bar denotes standard error at 95% confidence level..

Starch

Across all five sampling points, CK801 recorded the highest starch content in the roots; Suakoko 8 in the shoots. Supa and CK801 had the lowest starch content in the roots and shoots, respectively. At sampling point 1, the highest content of starch in both roots and shoots was noted in Suakoko 8. IR64 had the least in the roots and CK801 and IR64 had the least in the shoots. At sampling point 2, again, Suakoko 8 recorded the highest starch content for both tissues, whereas CK801 recorded the least. Observed at sampling point 3 was IR64's highest starch content in both roots and shoots. In comparison, CK801 had lower values for the roots and Supa, for the shoots. At sampling point 4, CK801 recorded a high accumulation of starch in the roots but the least starch content in the shoots. At sampling point 5, all genotypes showed relatively the same accumulation of starch in the roots with IR64 recording high amounts in the shoots (Fig.14d).

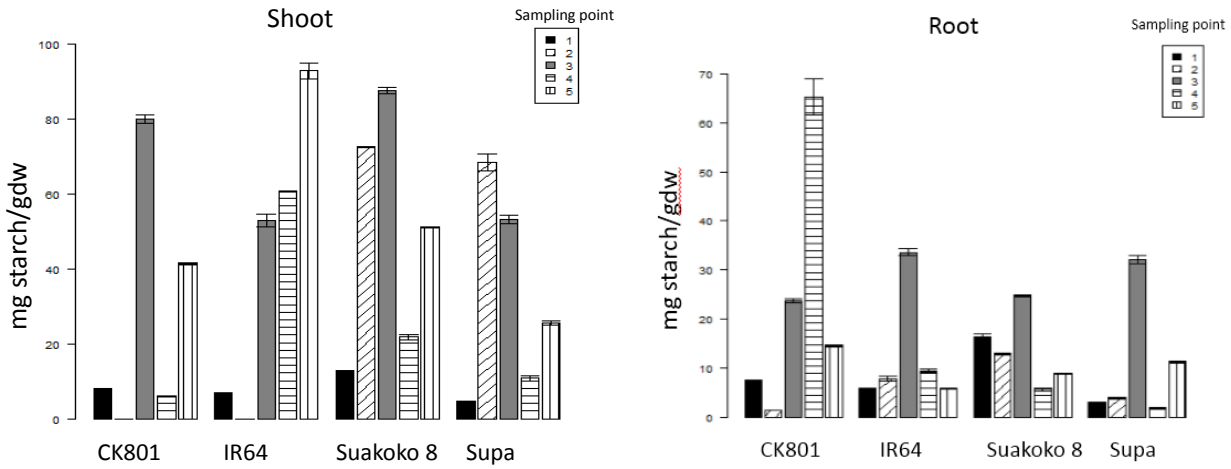


Fig14d: Relative starch accumulation in shoots and roots across sampling points, by genotype. Error bar denotes standard error at 95% confidence level

Lipid peroxidation

Levels of lipid peroxidation measured through quantification of MDA in both roots and shoots indicated almost the same trend for the tolerant and sensitive varieties. Sensitive genotypes IR64 and Supa recorded increasing levels of MDA from sampling points 1 to 2, with a slight decrease in sampling point 3, and a sharp increase in sampling point 5 thereafter. CK801 and Suakoko 8 maintained relatively low levels of MDA in both tissues at sampling points 1 to 4 with Suakoko 8 showing an increasing trend at sampling points 4-5 in the shoots (Fig.14e).

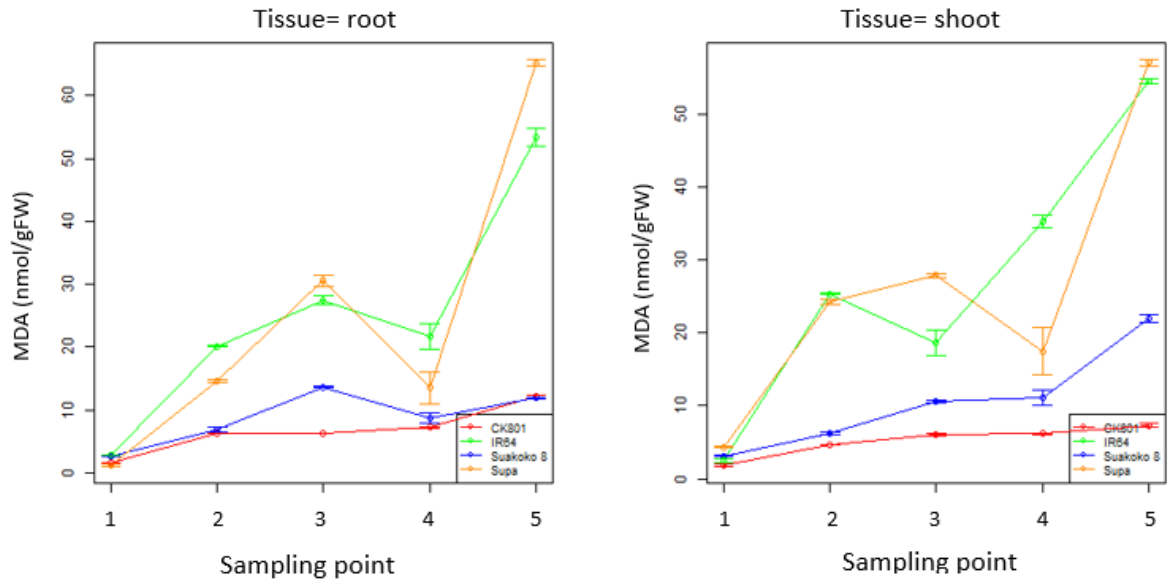


Fig14e: Levels of MDA across tissue, genotype and sampling points under Fe toxic conditions. Error bars denotes error bars at 95% confidence level

Total phenolic compounds (TPC)

CK801 recorded increasing levels of TPC from sampling 3 to 5 in both root and shoot. All genotypes except for Supa recorded very low TPC levels at sampling point 2 in both tissues. All genotypes recorded high levels of TPC in shoots compared to their roots across all sampling points. Suakoko 8 had the same pattern of TPC levels in both root and shoot. Supa was observed to maintain appreciable TPC levels in sampling 2 and 3 in both tissues. IR64 recorded increasing TPC levels in the shoot from sampling point 3 to 5 (Fig.14f).

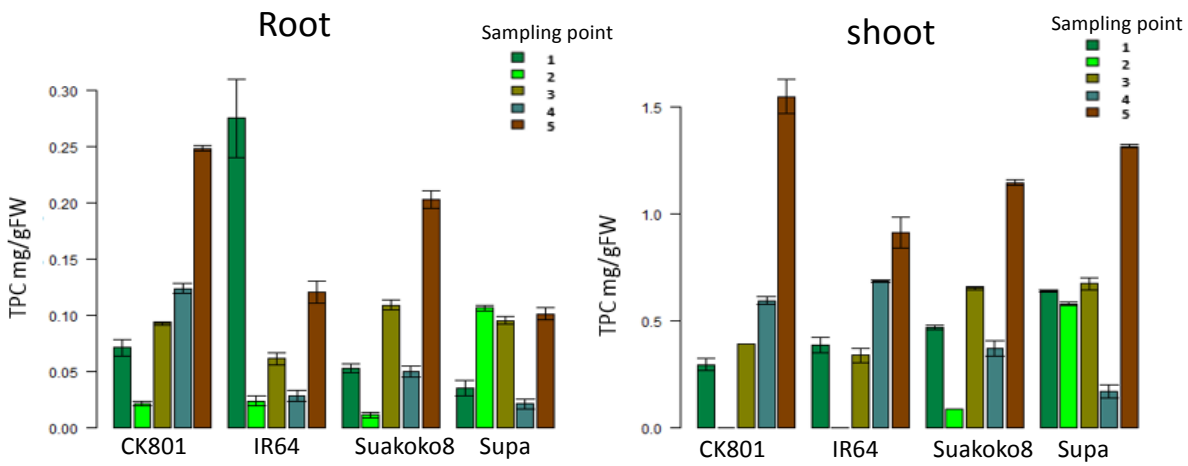


Fig14f: Levels of TPC across tissues, genotypes, and sampling points under Fe toxic conditions. Error bar denotes 95% confidence level.

4.2.3.3 Relative anti-oxidative activity and anti-oxidant content in plant tissues

Enzyme activities varied across all four genotypes, five sampling points, two tissue types, and Fe treatments. In the shoots, tolerant genotype CK801 recorded higher SOD activity than sensitive IR64, while Suakoko 8 and Supa had relatively the same activity for SOD. Catalase (CAT) activities were high for Suakoko 8 and Supa compared with those for CK801 and IR64. Ascorbate peroxidase (APX) activity was different for every genotype, with IR64 recording high activity across all sampling points and CK801 recording low activity for the same enzyme. Glutathione reductase (GR) was high in CK801 and relatively low in IR64, Suakoko 8, and Supa with slight differences among the three. Peroxidase (POX) activity was lower in CK801 compared with those in IR64, Suakoko 8, and Supa (Fig. 15a)

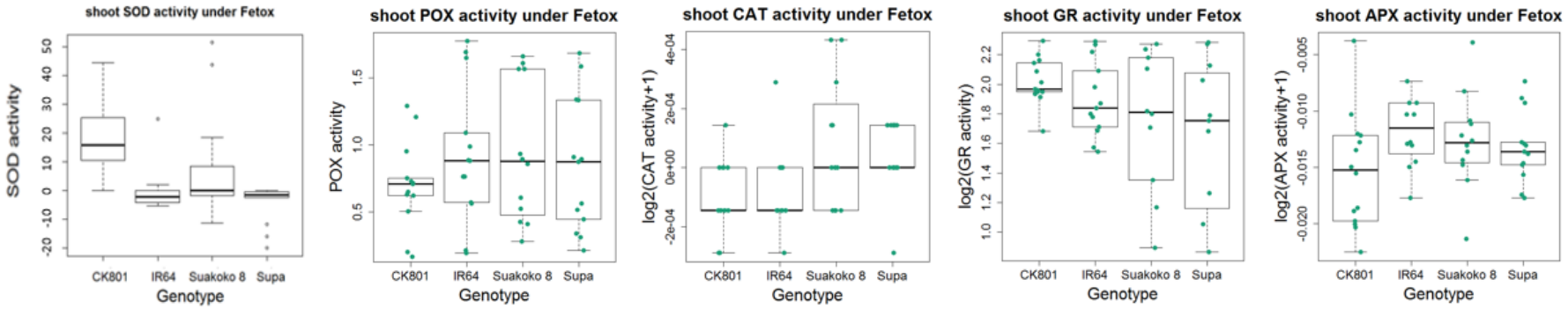


Fig 15a: Anti-oxidative enzyme activities in shoots of plants exposed to Fe toxicity. All enzyme activity is expressed as nmol/mg/ min

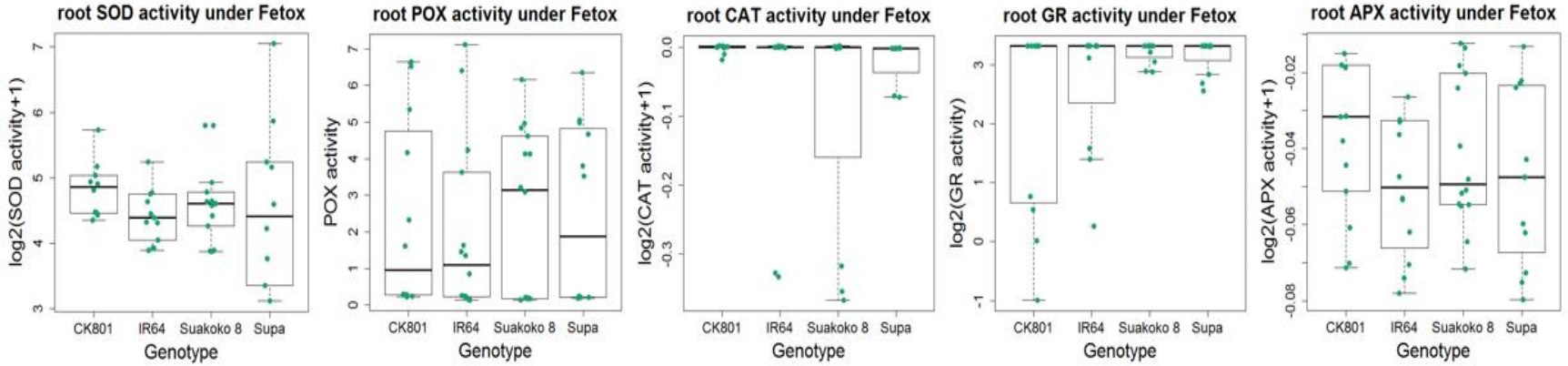


Fig 15b: Anti-oxidative enzyme activities in roots of plants exposed to Fe toxicity. All enzyme activity is expressed as nmol/mg/ mi

For the roots, enzyme activities varied among genotypes: CK801 had high enzyme activities for SOD, APX, and GR; Suakoko 8 recorded high enzyme activities for CAT and POX; IR64 showed low enzyme activities for SOD and POX; and Supa revealed intermediate enzyme activities for all enzymes (Fig.15b).

4.2.3.4 Anti-oxidative enzyme activities across time, treatments, genotypes, and tissues

All four genotypes used in this study showed variations in enzymatic activities across the sampling points. CK801 exhibited higher SOD activity both in the roots and shoots than did Suakoko 8, which recorded a drastic increase in the shoots and a drastic increase in the roots after sampling point 4. SOD activities were equally low for Supa in the roots as compared with those of IR64, which indicated fluctuations in its activities from one sampling point to another in both shoots and roots. A basin was also noted in Supa between sampling points 2 and 3 for SOD activity in the shoots (Fig.15c).

APX and CAT activities in the roots were inversely proportional to each other. For CK801 and IR64, low APX activities were observed at sampling points 1 to 3, followed by an exponential increase at sampling point 4 and a sharp drop afterward. Suakoko 8 recorded the lowest enzyme activity for APX across all the sampling points in the roots, followed by Supa. In the shoots, for all four genotypes, a high APX activity was recorded at sampling point 1, followed by unsteady pattern of decrease-increase-decrease (Fig. 15c).

CAT activity in the roots started with high values at sampling point 1 for all genotypes with CK801 maintaining a relatively constant activity across the five sampling points. Supa had a slight reduction in APX activity at sampling point 2, while Suakoko 8 and IR64 had a sharp drop

in their APX activity at sampling point 2, then another sharp increase at sampling point 3, after which they maintained a relatively high steady state of APX activity through to sampling point 5.

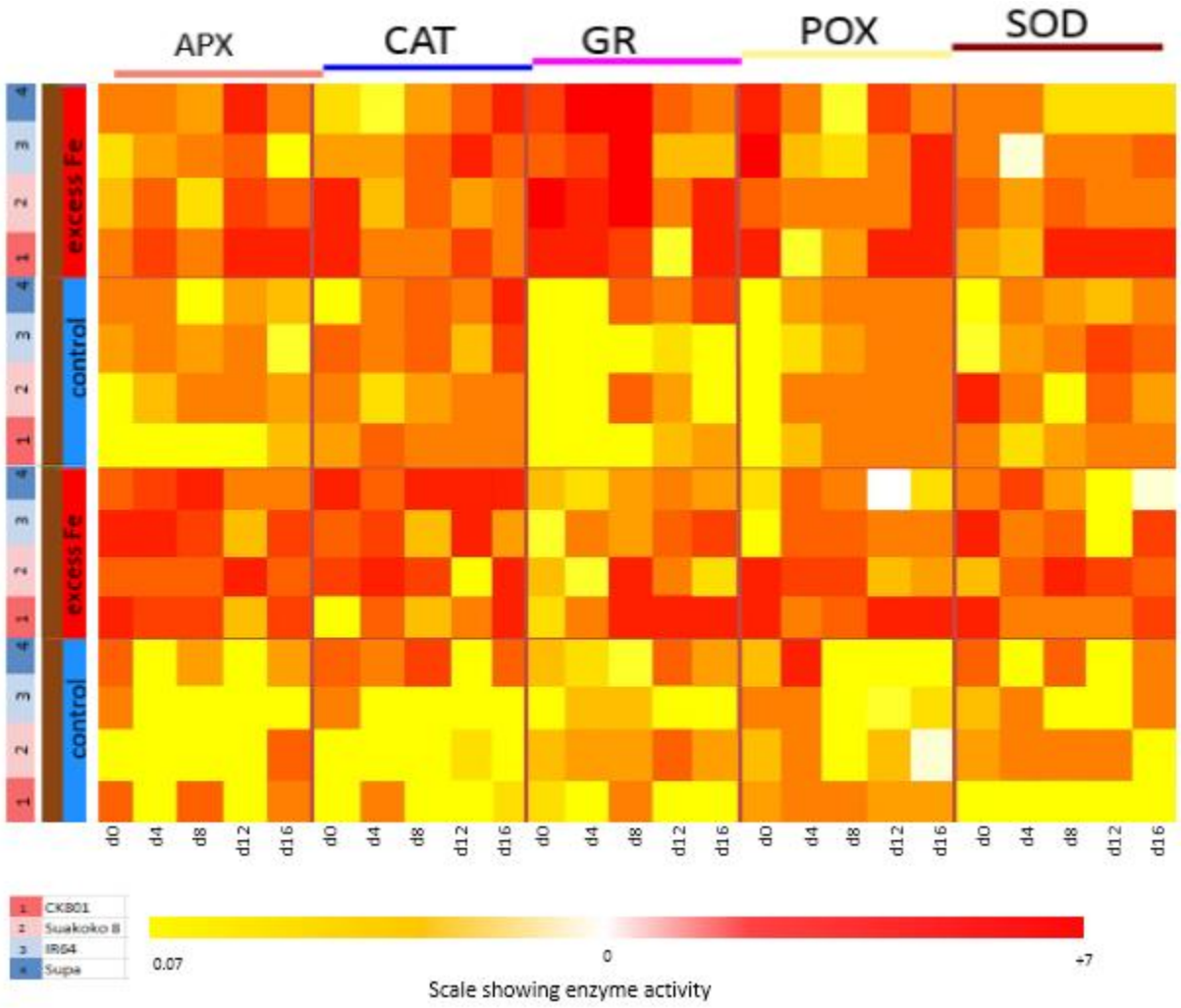


Fig15c:Heatmap of antioxidative enzymes' activities measured at different sampling points in the roots and shoots of the four varieties grown under control and excess Fe conditions. The white color means that no enzymatic activity was detected. d0 = 0 days, d4 = 4 days, d8 = 8 days, d12 = 12 days, d16 = 16 days of excess Fe treatment. APX= ascorbate peroxidase, CAT= catalase, GR= glutathione reductase, POX=peroxidase, SOD=superoxide dismutase

In the shoots, CAT activity was observed to decrease for IR64 and Supa, with IR64 recording the lowest values, while Suakoko 8 showed an appreciable increase in CAT activity from sampling points 1 through 4, after which CAT activity was seen to decrease. CK801 showed fluctuations in CAT activity, decreasing at sampling points 2 and 4 and increasing at sampling points 3 and 5 (Fig.15c).

POX activity in the roots formed a trough for all the genotypes, whereas in the shoots, POX activity was directly proportional to sampling point for all genotypes, aside from IR64, which started a decreasing trend after sampling point 4 (Fig. 15c).

All the genotypes maintained a steady GR activity in the roots from sampling points 1 to 3. GR activity drastically decreased at sampling point 4, with CK801 recording the least activity in sampling point 5. In the shoots, GR activity was high for all genotypes at sampling point 1. It decreased steadily across sampling points for Suakoko 8 and Supa, while CK801 and IR64 maintained a relatively high GR activity across all sampling points.

4.3 Histochemical analyses

4.3.1 Aerenchyma quantification

Poor aerenchyma development was visible in Supa from the imaged hand sections. IR64 exhibited well-developed aerenchyma and so did Suakoko 8 and CK801 (Fig.16a). Root and stele diameters were also evidently different in all genotypes with CK801 recording the high root and stele diameters. No significant difference was recorded for % aerenchyma in CK801 and Suakoko 8. However, Supa recorded reduced counts of aerenchyma as compared with IR64 whose root and stele diameters were lowest among all genotypes (Table 10).

Table 10: Quantification of aerenchyma from roots under Fe toxicity treatment

Genotype	Root diameter (pixels)	Stele diameter (pixels)	Aerenchyma (pixels)	% aerenchyma
CK801	474198	57805	134478	32.30
Supa	361498	42871	49694	15.60
IR64	282199	32894	60239	24.16
Suakoko 8	385871	41180	111801	32.44

Better aerenchyma development was observed at the root cap of tolerant genotype Suakoko 8 as compared with those seen in other genotypes. However, aerenchyma quantification at the root base did not indicate any significant difference in % aerenchyma between CK801 and Suakoko 8. Supa gave the lowest percentage of % aerenchyma of the four genotypes (Fig. 16a).

4.3.2 Aerenchyma, Lignin and suberin observations

Microscopic examinations of lignin and suberin indicated more suberization and lignification in tolerant Suakoko 8 than in CK801 and sensitive genotypes IR64 and Supa. In the two tolerant genotypes, lignin and suberin deposition was obviously visible both in the root base (5-10 mm from the shoot), mid-root (10-15 mm from the root tip), and root cap (0-5 mm from the root tip). However, density of lignification and suberization was higher at the endodermis (inner root) as well as in the sclerenchyma (outer root) of Suakoko 8 as compared with that of other genotypes (Fig. 16b).The sensitive genotypes also showed relatively good accumulation of lignin and suberin at the sclerenchyma and endodermis at the root base, but this was not observed at the root cap and mid root (Fig. 16b).

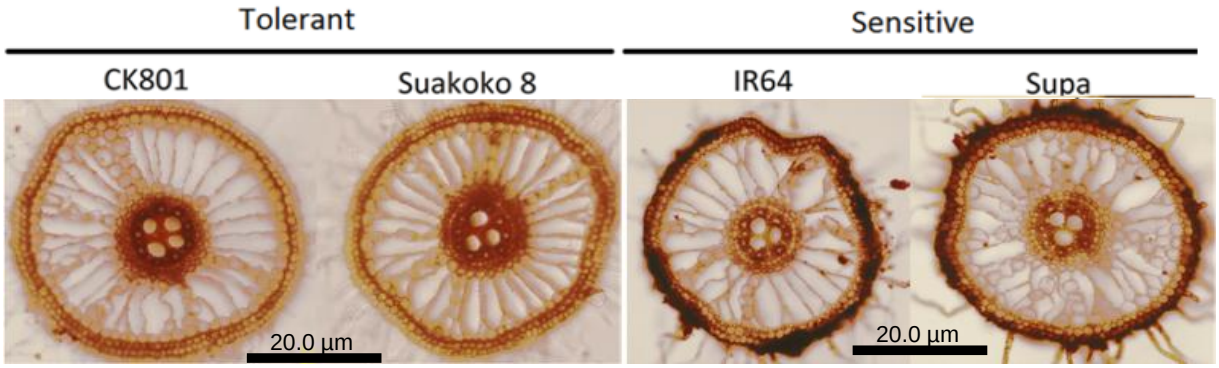


Fig 16a: Microscopic images of root base (5-10 mm from the shoot) cross-sections of the 4 genotypes stained with Sudan red for suberin; red stains show suberin deposits both at the endodermis and exodermis and aerenchyma development.

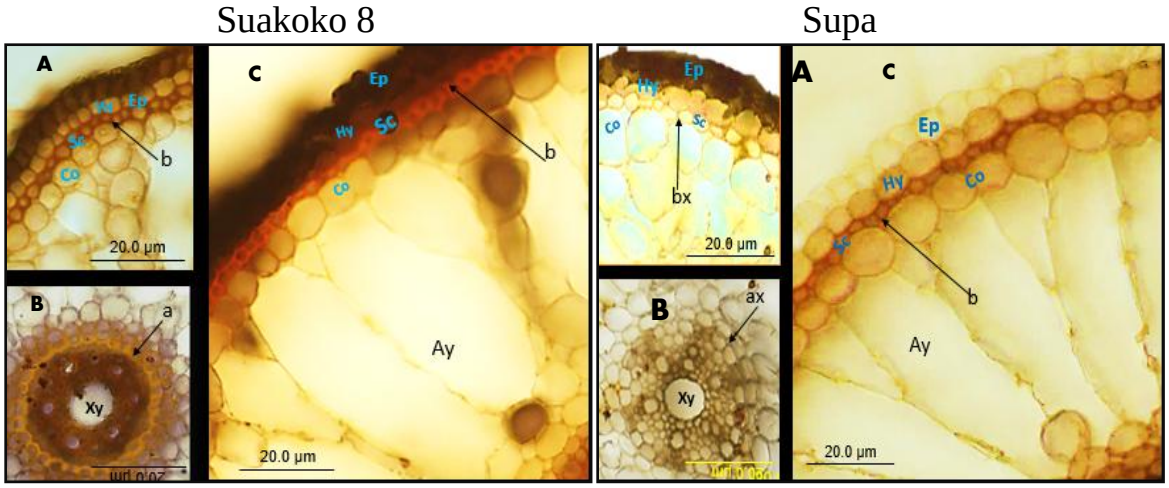


Fig 16b: Microscopic images of root cap (0-5 mm from the tip) (A) central cylinder of the root cap (B) root base (5-10 mm from the shoot) (C) cross-sections stained with phloroglucinol- HCl for lignin and Sudan red for suberin. Ep-epidermis; Hy-hypodermis/exodermis; Sc-sclerenchyma; Co-cortex; a-lignified endodermis; ax-unlignified endodermis; b- suberin deposition; bx- no suberin deposition; Ay- Aerenchyma; xy- xylem.

Lignin accumulation was visible in the stem base (1-5 mm from the root) of all the genotypes and so was good aerenchyma development (image not shown). Leaf cross-sections did show significant differences in lignin and suberin deposition among the genotypes. Suakoko 8 had

suberin deposits on both the adaxial and abaxial leaf surfaces, while suberin deposition in CK801 was only observed in the adaxial leaf surface. For the sensitive varieties, no suberization was observed on both sides of the leaf (Fig.16c). Lignification was however observed in all the genotypes around the vascular bundles.

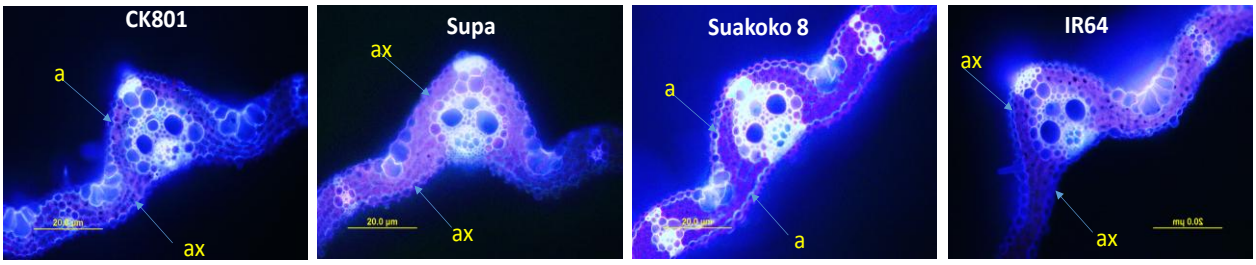


Fig16c: Microscopic images of leaf cross-sections stained with Sudan red for Suberin is visualized at the adaxial and abaxial leaf as an auto-fluorescence of light blue. a -suberin deposition; ax-no suberin deposition

4.3.3 Root Oxidation Power

Radial oxygen loss using the reduction and oxidation of methylene blue did not show any significant difference between CK801 and IR64 both at day 10 and day 14 of excess Fe^{2+} treatment. However, increasing radial oxygen loss was very significant between Suakoko 8 and Supa across time at day 10 and between day 10 and day 14 (Fig.17).

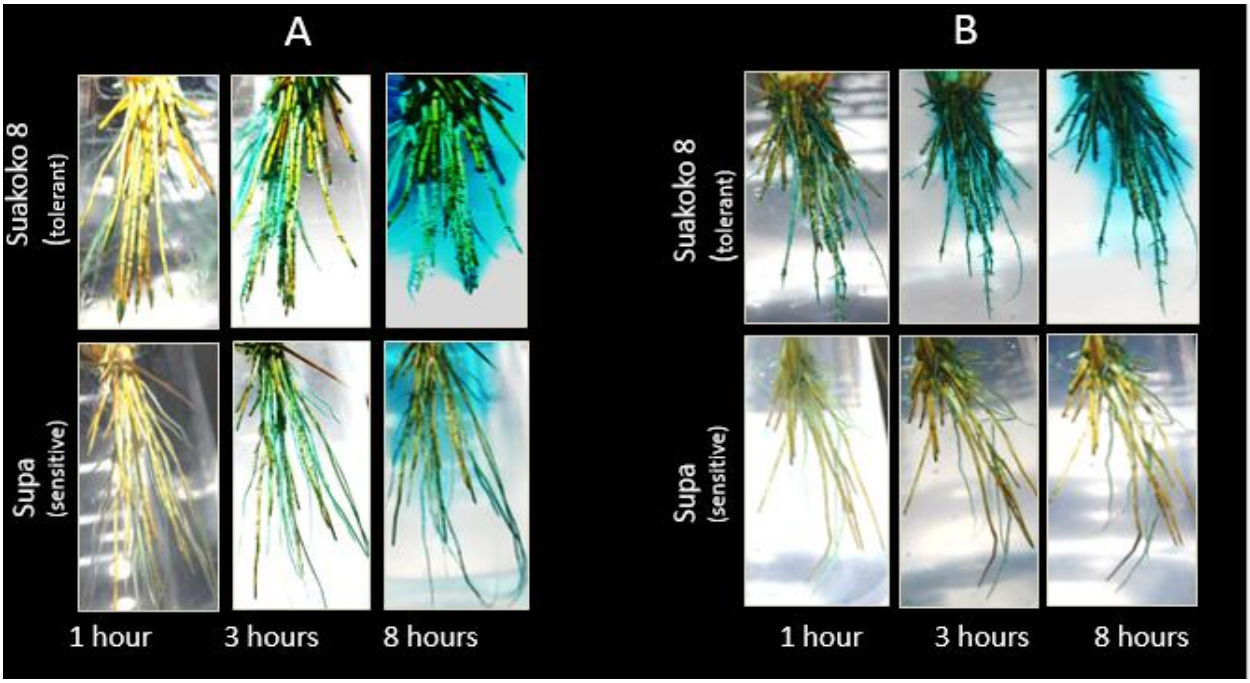


*Fig 17: Radial oxygen loss in tolerant and sensitive lowland rice variety after 10 days (**A**) and 14 days (**B**) of excess Fe^{2+} (300ppm) treatment in hydroponics*

After 8 hours of the setup, a blue mass of oxidized methylene blue was observed surrounding the root system of Suakoko 8 as opposed to Supa in both day 10 and day 12. At day 12 however, very little changes in solution colour was noted for Supa whose most lateral roots were already dead.

4.4 Gene Expression analysis

4.4.1 Phenotypic response of two rice plants exposed to excess iron stress

Differential phenotypic response was observed in the varieties over time indicating varying levels of iron toxicity tolerance with time. Time and variety was significant $P<0.05$ across all growth parameters. Interaction between time x treatment was equally significant $P< 0.05$ across

all parameters. However interaction between time x variety x treatment was only significant P<0.05 for number of green and dry leaves (Table 11a).

Table 11a: Varietal response to iron treatment, stress duration (time) and their interactions on the morphological traits measured

	shoot height(cm)	leaf length (cm)	no. green leaves	no. dry leaves	leaf width (cm)	no. tillers	Root length (cm)	no. lateral roots
Time	****	****	****	****	****	**	*	****
treatment	****	****	****	****	****	***	ns	Ns
Variety	****	****	****	**	**	****	*	*
Time: treatment	****	***	****	****	***	**	*	*
Time: Variety	ns	ns	ns	****	ns	***	ns	Ns
treatment: Variety	**	ns	****	***	ns	****	ns	Ns
Time: treatment: Variety	ns	ns	*	****	ns	***	ns	Ns

***** P<0.0001; ***P<0.001; **P<0.01; *P<0.05; ns= not significant*

Despite the varying significance observed for time x variety, growth was observed in both varieties for most traits from the day of excess Fe^{2+} treatment to the sixth day when a decrease was observed in shoot height and number of functional lateral roots in both varieties. However, increase in leaf length was observed in both varieties up to day ten of excess Fe^{2+} treatment, increase in number of green leaves was observed in CK801 with concomitant decrease in Supa between day eight and ten. This observation was tandem with a drastic increase in number of dried leaves in Supa between day eight and ten. No significant difference was observed in the root length growth between the two varieties. (Fig. 18).

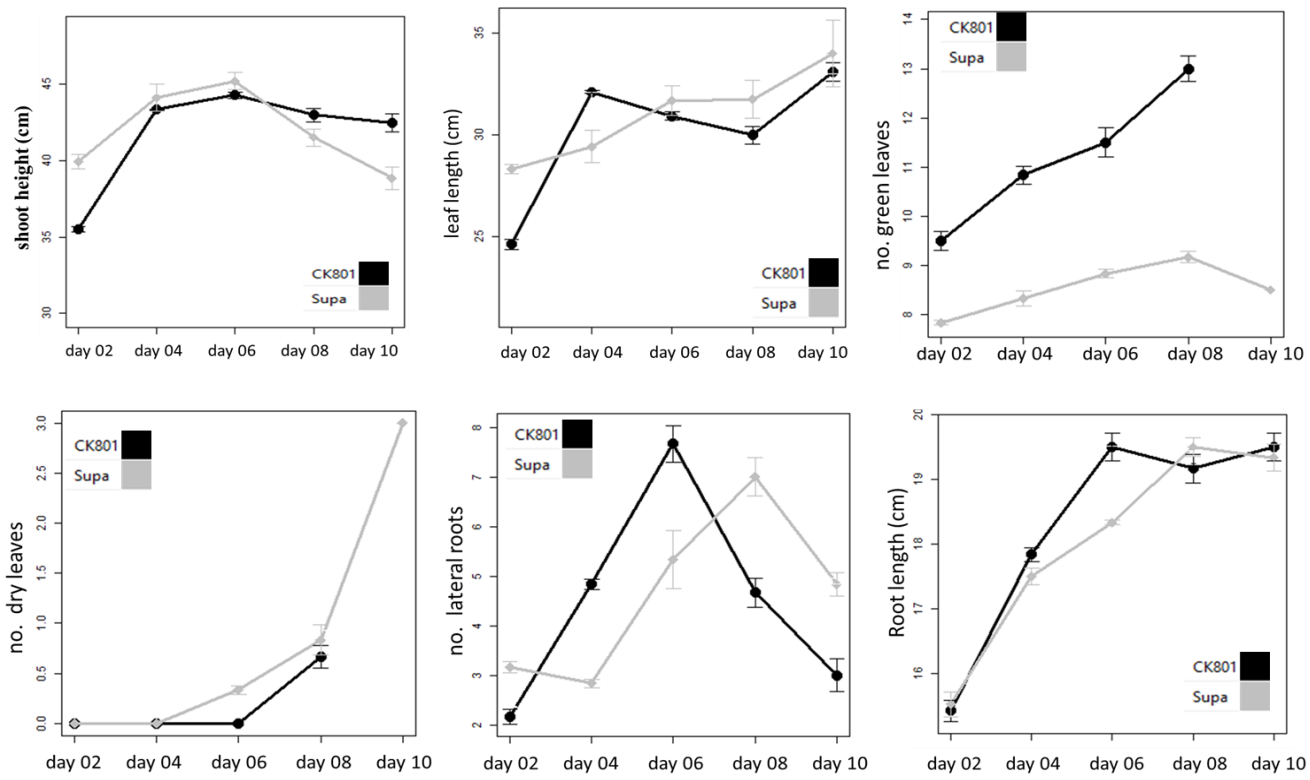


Fig 18: Varietal morphological performance over time under excess Fe2+ (300ppm) treatment

4.4.2 Quantification of %Fe in the tissues of two rice genotypes

The quantification of %Fe was realized in both shoot and roots. Excess iron stress induced Fe accumulation in both root and shoots of both varieties compared to the control after 14 days of Fe treatment with Supa recording high percentage in both root and shoot compared to CK801 at (300ppm). However, significantly high amounts of %Fe was accumulated in the roots compared to shoots under control (2ppm) (Table 11b).

Table 11b: %Fe accumulation in root and shoot of Fe^{2+} treated plants after 14 days of treatment

Tissue	**Variety**	**%Fe on dried ground tissues**	
		Fe^{2+} (300ppm)	Fe^{2+} (2ppm)
Root	*CK 801*	4.12**	0.18 ns
	Supa	5.23**	0.19 ns
Shoot	*CK 801*	0.18*	0.03ns
	Supa	0.35*	0.02 ns

***P<0.01; *P<0.05; ns= not significant*

4.4.3 Expression of genes involved in DMA biosynthesis and transport for Fe (III)-DMA uptake/translocation

Differential expression of genes was evident with tissue sampling data and variety. Zero upregulation was recorded in all the 6 genes (*OsSAMS2, OsNAS1, OsNAAT1, OsDMAS1, TOM1 (OsZIFL4), OsYSL15*) responsible for DMA biosynthesis and transport for Fe (III)-DMA uptake/translocation at day 3 of Fe treatment in the roots of Supa and shoots of CK801. Only one gene *OsSAMS2* was upregulated in the roots of CK801 on day 3. However, up to 5 genes were upregulated in the shoot of Supa with the exception of *OsYSL15* on day 3. On day 7 of Fe treatment 5 genes responsible for DMA biosynthesis were upregulated in the roots and shoots of both CK801 and Supa with *OsSAMS2* being the only gene down regulated in both varieties and in both tissues. Highest upregulation was recorded for in the roots of Supa at day 7 of Fe treatment with the gene *OsYSL15* (Summary table 12 and Figure19a).

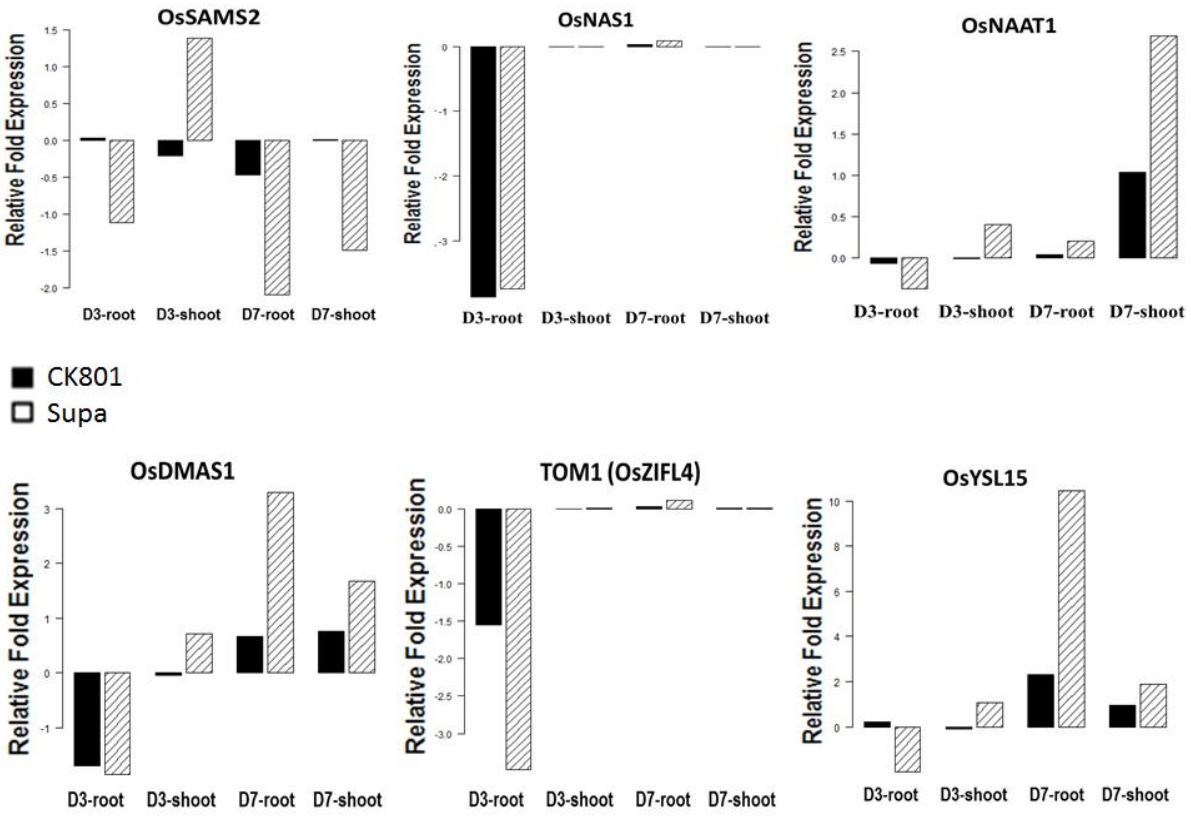


Fig 19a: Genes involved in DMA biosynthesis and transport for Fe(III)-DMA uptake/translocation. D3=day 3 of excess Fe^{2+} treatment and D7= day 7 of excess Fe^{2+} treatment.

4.4.4 Expression of transporters and related genes for ferrous Fe uptake/translocation

At day 3 of Fe treatment, genes involved in the uptake/translocation of ferrous (*OsFRO1, OsFRO2, OsIRT1, OsNRAMP1*), were all down regulated in roots of the two test varieties with an upregulation being recorded for in both varieties for gene *OsFRO1* in the shoots, *OsNRAMP1* in CK801 and *OsIRT1* in Supa both in the shoot at day 3 of Fe treatment. At day 7 of Fe treatment, all the four genes involved in ferrous Fe uptake/translocation were upregulated in the roots of both varieties and in the shoot of CK801 with a concomitant down regulation in the shoots of Supa. (Summary table 12 and Fig 19b).

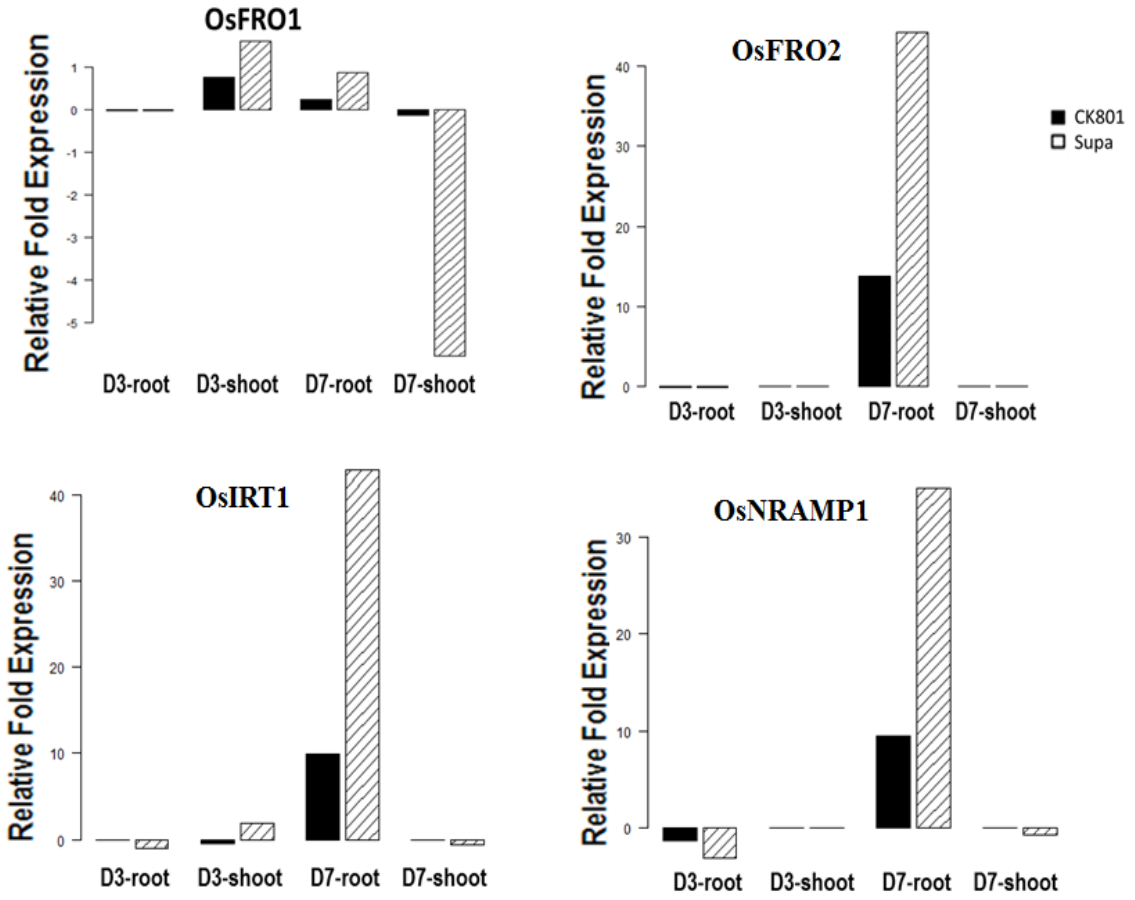


Fig 19b: Expression of transporters and related genes for ferrous Fe uptake/translocation. D3=day 3 of excess Fe^{2+} treatment and D7= day 7 of excess Fe^{2+} treatment.

4.4.5 Expression of transporters for Fe translocation genes

Transporters for Fe translocation genes (*OsYSL18*, *OsFRDL1*, *PEZ2*, and *OsYSL2*) were all down regulated in the root of CK801 at day 3 and all except*OsFRDL1* upregulated in its roots at day 7. However, in the shoots of CK801 only one gene *OsYSL2* was down regulated at day 3 of Fe treatment in the shoots with all the four genes being upregulated in the shoots of CK801 at day 7 of Fe treatment. At day 3, all genes except *OsYSL2* and *OsYSL18* were down regulated in the root and shoots of Supa respectively. While at day 7 of Fe treatment three genes *OsYSL18, PEZ2, OsYSL2* and one gene *OsYSL2* were all upregulated in the roots and shoots of Supa respectively (Summary table 12 and Fig.19c).

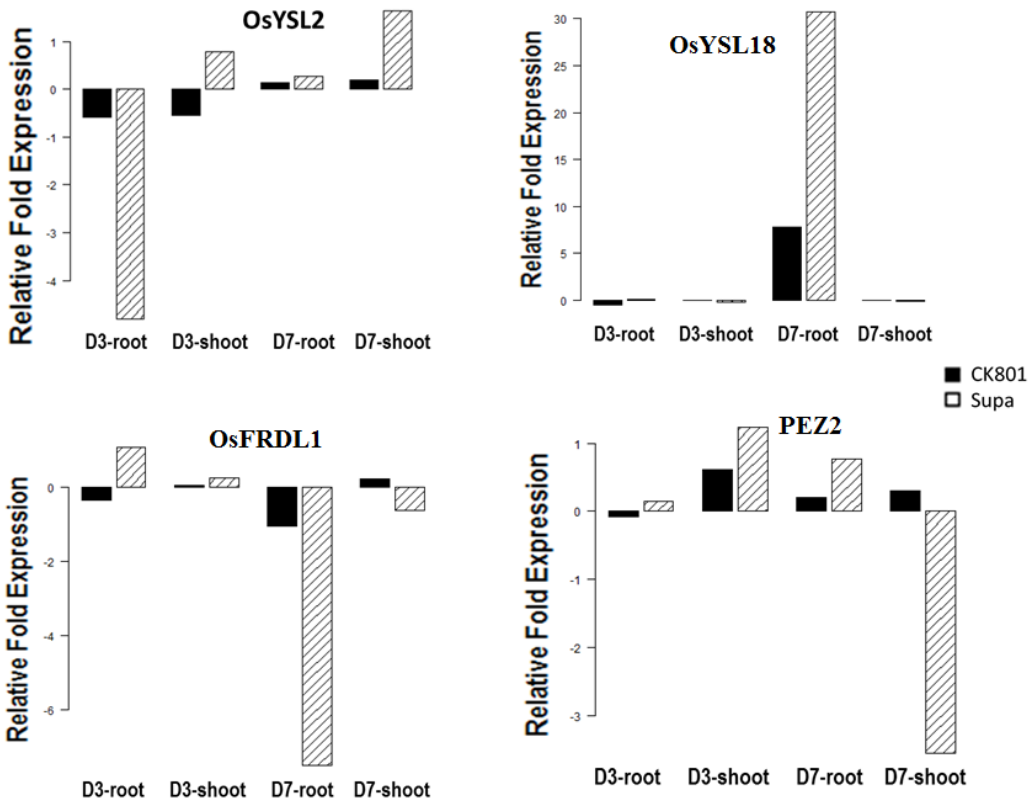


Fig 19c: Expression of transporters for Fe translocation genes. D3=day 3 of excess Fe^{2+} treatment and D7= day 7 of excess Fe^{2+} treatment.

4.4.6 Expression of transporters and chelators for subcellular storage / Fe sequestration

All the six (*OsVIT2, OsMIT, OsMIR, TIC21, OsFer1,OsFer2*) genes involved in transport and chelation for subcellular storage/Fe sequestration were all upregulated in the roots of both varieties at day 7. At day 3, *OsVIT2, OsFer1* and*OsFer2* were upregulated in the roots and shoots of both varieties. *OsMIT*, *OsMIR* and *TIC21* were down regulated in the root and shoot of CK801 at day 3 and in the shoots of Supa at day 7. High upregulation was recorded with gene *OsMIR* in roots of Supa at day 7 (Summary table12 and Fig 19d).

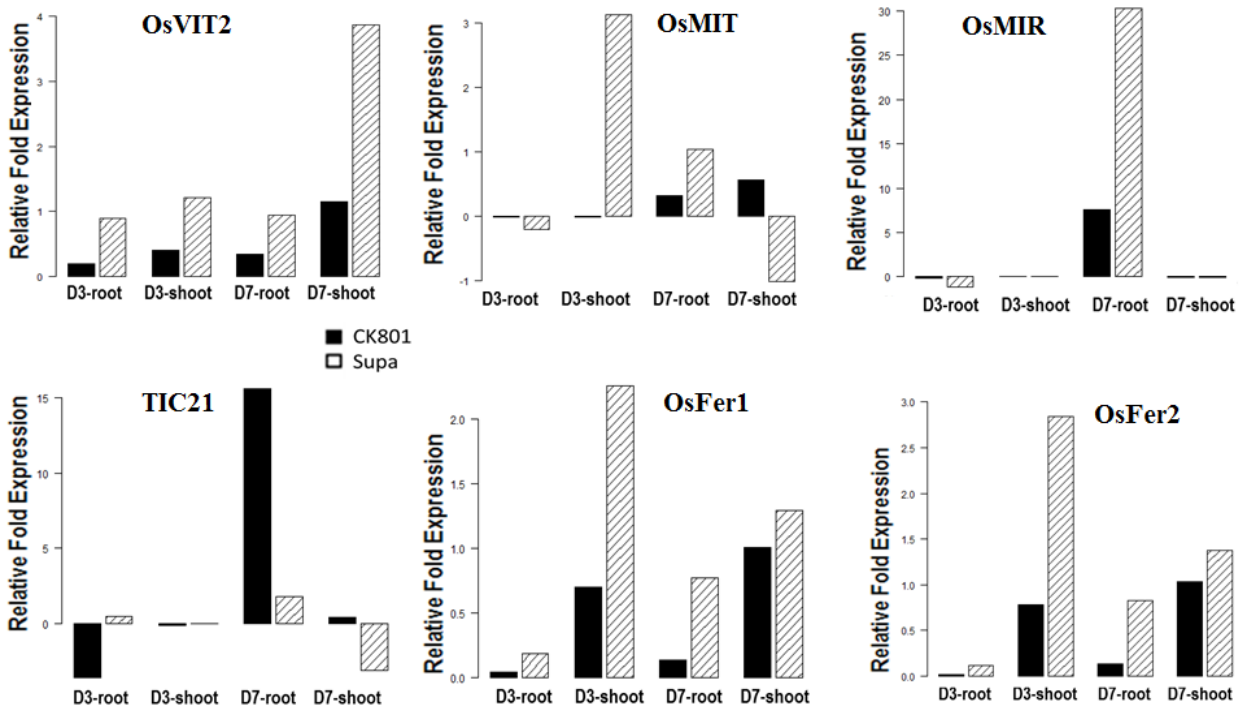


Fig 19d: Expression of transporters and chelators for subcellular storage / Fe sequestration. D3=day 3 of excess Fe^{2+} treatment and D7= day 7 of excess Fe^{2+} treatment.

4.4.7 Expression of genes involved in Iron homeostasis regulation

Upregulation was recorded with all iron homeostasis regulation genes (*OsIRO2, OsIRO3, OsbHLH133*, and *OsIDEF1)* in the roots of both varieties and in the shoots of CK801 at day 7 of Fe treatment. However, a down regulation was recorded for in CK801with all the four genes involved in the regulation of Fe homeostasis at day 3 of Fe treatment. In Supa all except OsIDEF1 was down regulated in roots and all except *OsIRO2* upregulated in shoots at day 3 of Fe treatment. High upregulation was recorded with gene *OsbHLH133* in the root of Supa at day 7 of Fe treatment (Summary table 12 and Fig.19e).

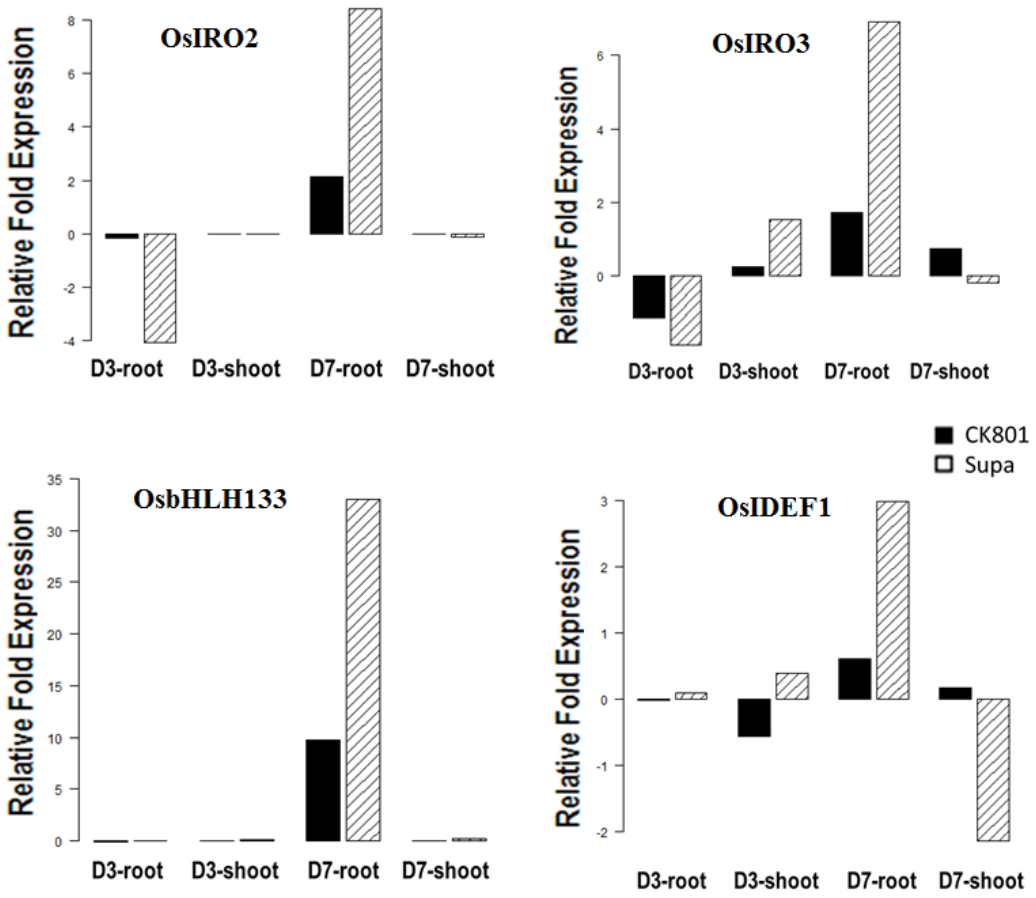


Fig 19e: Expression of genes involved in Iron homeostasis regulation. D3=day 3 of excess Fe^{2+} treatment and D7= day 7 of excess Fe^{2+} treatment.

4.4.8 Expression of genes involved in protection from oxidative damage by ROS

Most of the genes involved in protecting plant against oxidative damage by ROS were upregulated in both varieties with high upregulation recorded for in the roots of Supa at day 7 in Glutathione-S-transferase (GST-(LOC_Os01g49720)). Glutathione transporter was down regulated in both varieties and tissues except in the roots of Supa at day 3 and upregulated in both varieties and tissues except in the shoots of Supa at day 7. GST was upregulated in all tissues, all varieties and all days. Expression of Peroxidase (OsPx4) was upregulated in the roots of CK801 both days and down regulated in the shoots of Supa in both days. Peroxidase (POX) which is a precursor, related to lignin biosynthesis was upregulated in all tissues in both varieties in all days of Fe treatment. Catalase isozyme A (*OsCAT-A*) was down regulated in the roots and

upregulated in the shoots of CK801 in both days of Fe treatment. Fe-SOD (sodB) which is involved in the conversion of superoxide radicals to molecular oxygen was down regulated in both varieties and tissues at day 3 of Fe treatment and up regulated in the roots of both varieties and shoots of CK801 at day 7 of Fe treatment. Mn-SOD was up regulated in all tissues in both varieties and days except for the shoots of CK801 at day 3 of Fe treatment. Cu/Zn-SOD was upregulated in both tissues in both varieties in all days except for shoots of Supa in day 7. Glutathione reductase was upregulated in all tissues in both varieties and both days of Fe treatment (Summary table 12).

Other genes involved in iron regulation that were upregulated in the roots of both varieties at day 7 of Fe treatment include; Auxin efflux carrier Transporter, No apical meristem protein, Beclin-1, Potassium channel *AKT1* and Methyltransferase (Summary table 12).

Table 12: Expression of genes involved in iron uptake and homeostasis in rice roots and shoots after 3 and 7 days of excess $Fe2^{+}$ (300ppm) treatment in hydroponics

		Day 3				Day 7			
		Root		Shoot		Root		Shoot	
GENE NAME	FUNCTION	CK801	Supa	CK801	Supa	CK801	Supa	CK801	Supa
DMA biosynthesis and transport for Fe(III)-DMA uptake/translocation									
OsSAMS2	S-adenosyl-L-methionine synthetase (SAM = NA & ethylene precursor)	0.037	-1.110	-0.200	1.391	-0.457	-2.093	-0.008	-1.494
OsNAS1	Nicotianamine synthase	-3.874	-3.745	-0.001	0.000	0.030	0.086	0.000	0.000
OsNAAT1	Nicotianamine aminotransferase	-0.065	-0.370	-0.009	0.412	0.044	0.207	1.170	2.691
OsDMAS1	Deoxymugineic acid synthase	-1.695	-1.858	-0.051	0.705	0.664	3.285	0.749	1.676
TOM1 (OsZIFL4)	DMA efflux transporter	-1.550	-3.482	-0.002	0.005	0.032	0.115	0.005	0.011
OsYSL15	Fe(III)-DMA transporter from the rhizosphere to roots	0.215	-1.994	-0.082	1.058	2.305	10.430	0.961	1.876
Transporters and related genes for ferrous Fe uptake/translocation									
OsFRO1	Ferric chelate reductase, reduces Fe3+ into Fe2+	-0.012	-0.015	0.759	1.619	0.254	0.884	0.169	-5.793
OsFRO2	Ferric chelate reductase, reduces Fe3+ into Fe2+	-0.142	-0.071	-0.055	-0.055	13.746	44.235	0.002	-0.020
OsIRT1	Iron-regulated transporter 1, Ferrous Fe transporter	-0.101	-1.014	-0.503	1.897	9.859	42.775	0.000	-0.659
OsNRAMP1	Ferrous Fe transporter	-1.270	-3.108	0.047	-0.039	9.436	35.014	0.033	-0.727
Transporters for Fe translocation									
OsYSL2	Fe(II)/manganese(II)-NA transporter	-0.590	-4.810	-0.539	0.787	0.134	0.265	0.959	1.643
OsYSL18	Fe(III)–DMA transporter, Fe distribution in reproductive organs, lamina joints, and phloem cells at the base of the leaf sheath	-0.491	0.105	0.014	-0.223	7.784	30.744	0.038	-0.054
OsFRDL1	Ferric reductase defective 3 like ,Citrate efflux transporter	-0.369	1.064	0.030	0.230	-1.050	-7.488	0.206	-0.644
PEZ2	Phenolics efflux zero 2, involved in solubilization of apoplasmic Fe in rice	-0.083	0.152	0.607	1.229	0.203	0.771	0.305	-3.547
Transporters and chelators for subcellular storage / Fe sequestration									
OsVIT2	Vacuolar iron transporter 2, Fe transporter into vacuole and sequestration	0.191	0.889	0.408	1.207	0.340	0.939	1.154	3.868
OsMIT	Mitochondrial Fe transporter, Fe transporter into mitochondria	-0.007	-0.203	-0.004	3.134	0.323	1.038	0.573	-1.012
OsMIR	Mitochondrial iron-regulated gene	-0.139	-1.175	-0.010	-0.024	7.566	30.302	-0.027	-0.091
TIC21	Permease in chloroplasts 1, Fe transporter into chloroplast	-3.550	0.524	-0.109	0.024	15.585	1.811	0.465	-3.066
OsFer1	Ferritin gene	0.042	0.184	0.700	2.256	0.136	0.771	1.010	1.296
OsFer2	Ferritin gene	0.023	0.122	0.786	2.841	0.140	0.828	1.038	1.378

Relative gene expression expressed as ΔΔCt of target and reference genes; Blue indicates down regulation (negative expression); Red indicates upregulation (positive expression)

Table 12 con't. Expression of genes involved in iron uptake and homeostasis in rice roots and shoots after 3 and 7 days of excess Fe2+ (300ppm) treatment in hydroponics

		Day 3				Day 7			
		Root		Shoot		Root		Shoot	
GENE NAME	**FUNCTION**	**CK801**	**Supa**	**CK801**	**Supa**	**CK801**	**Supa**	**CK801**	**Supa**
Iron homeostasis gene regulation									
OsIRO2	bHLH transcription factor, Positive transcriptional regulator	-0.175	-4.060	-0.005	-0.021	2.119	8.386	0.000	-0.116
OsIRO3	bHLH TF, Negative transcriptional regulator,	-1.139	-1.871	0.243	1.528	1.739	6.909	0.753	-0.178
OsbHLH133	Negative transcriptional regulator	-0.121	-0.029	-0.003	0.073	9.711	33.007	0.041	0.176
OsIDEF1	Iron deficiency-responsive element (IDE) factor 1	-0.017	0.088	-0.556	0.387	0.605	2.990	0.174	-2.140
Protection from oxidative damage by ROS									
Glutathione transporter	Glutathione transporter	-0.017	0.923	-0.252	-1.178	0.162	3.117	0.046	-0.548
GST	Glutathione-S-transferase	0.029	4.138	-0.175	0.303	-0.206	14.602	0.907	5.613
GST	Glutathione-S-transferase	0.228	0.928	0.668	1.370	7.453	26.703	1.534	6.544
OsPx4-a	Peroxidase	0.027	-0.025	-0.897	-0.514	0.130	0.455	0.012	-1.803
Peroxidase (POX)	Peroxidase precursor, related to lignin biosynthesis	0.042	0.184	0.700	2.256	0.136	0.771	1.010	1.296
OsCAT-A	Catalase isozyme A (CAT-A), catalyzes the decomposition of hydrogen peroxide to water and oxygen	-0.022	1.658	1.706	3.763	-0.056	-0.706	2.556	-1.543
OsCAT-B	Catalase isozyme B (CAT-B)	0.230	2.678	0.078	-0.173	0.571	2.589	1.296	3.988
OsCAT-C	Catalase isozyme B (CAT-C)	-0.021	-0.004	-0.532	-0.607	0.443	2.081	-0.008	-2.284
Fe-SOD (sodB)	Chloroplastic superoxide dismutase, catalyse the conversion of superoxide radicals to molecular oxygen	-0.102	-0.014	-0.660	-0.962	0.181	0.919	0.411	-3.445
Mn-SOD	Manganese-superoxide dismutase precursor	0.118	0.362	-0.283	1.418	0.294	0.833	0.982	1.353
Cu/Zn-SOD		0.019	0.046	2.049	6.042	0.612	1.991	1.483	-0.660
sAPX (L-APX 7)	stromal ascorbate peroxidase	-0.017	-0.005	1.580	5.050	1.502	1.007	2.879	1.330
tAPX (L-APX 8)	thylakoid-bound ascorbate peroxidase	-0.012	-0.023	-0.611	0.295	0.030	-0.039	-0.209	-5.181
cAPX (OsAPXb, L-APX 2)	cytosolic ascorbate peroxidase	-0.040	0.120	-0.518	-0.020	0.201	0.372	0.458	0.570
AO	L-ascorbate oxidase, multicopper oxidase family	0.708	-0.119	-0.070	0.015	2.460	4.818	0.287	-0.134
GR	Glutathione reductase, cytosolic	0.033	0.424	0.350	0.383	0.236	0.884	0.902	2.091
Other CG of interest									
Auxin efflux carrier Tansporter	transpot of auxin	0.004	0.086	-0.393	3.806	1.740	23.241	-0.341	-6.327
Potassium channel AKT1	Potassium transport	0.215	-1.994	-0.082	1.058	2.305	10.430	0.961	1.876
No apical meristem protein	Transcription factor, gene regulation	-0.340	-0.438	-0.700	0.836	2.136	6.632	0.146	-2.471
Beclin-1	similar to autophagy protein	1.935	2.557	-1.932	-3.181	0.061	0.537	0.005	-0.002
Methyltransferase	DNA methylation, gene regulation	1.935	2.557	-1.932	-3.181	0.061	0.537	0.005	-0.002

Relative gene expression expressed as ΔΔCt of target and reference genes; Blue indicates down regulation (negative expression); Red indicates upregulation (positive expression)

4.5 QTL mapping

4.5.1 QTLs identified using the Supa/CK801 population

Linkage analysis was performed with microsatellite genotyping data from the 1071 SNP markers using QGene v.4.3.2 (Nelson, 1997). Significant QTLs for root dry weight were identified on chromosomes 2 (LOD = 3.139; 3.043; 3.098), 4 (LOD = 3.217) and 9 (LOD=3.307; 3.326) while

QTLs for greenleavesatdaysixoftoxicirontreatment were identified on chromosomes 4 (LOD=3.018) and 10 (LOD=3.109) (Table 13; Fig 20). Figure 20 shows the distribution of the 1071 markers throughout the rice genome.

Table 13. QTLs identified from a population of 167 Supa / CK801 F2:3 using 1071 SNP markers and 16 iron toxicity tolerance traits measured at the seedling stage with a hydroponic system. Significant QTLs were identified for root dry weight (RDW) and green leaves at day six of toxic iron treatment (GLD6)

Traits	**Chr**	**QTL name**	**Nearest marker**	**QTL start**	**QTL stop**	**Peak LOD**	**Increased effect**	**R^2 (%)**
RootDW	2	RDW 2.1	id2006795	1905884	1935818	3.043	-0.294	8.2
	2	RDW 2.1	2120623	2087388	id2010102	3.139	-0.307	8.2
	2	RDW 2.1	2193623	2165278	2224585	3.098	-0.304	8.2
	4	RDW 4.1	4667490	4647616	4678550	3.217	-0.293	8.4
	9	RDW 9.1	9191137	9186082	9234177	3.326	-0.306	8.7
	9	RDW 9.1	9361710	id9002027	9409284	3.307	-0.315	8.7
GLD6	4	GLD6 4.1	3598944	3598944	3619998	3.018	-0.682	7.6
	10	GLD6 10.1	id10000153	id10000153	9990622	3.109	-0.533	7.8

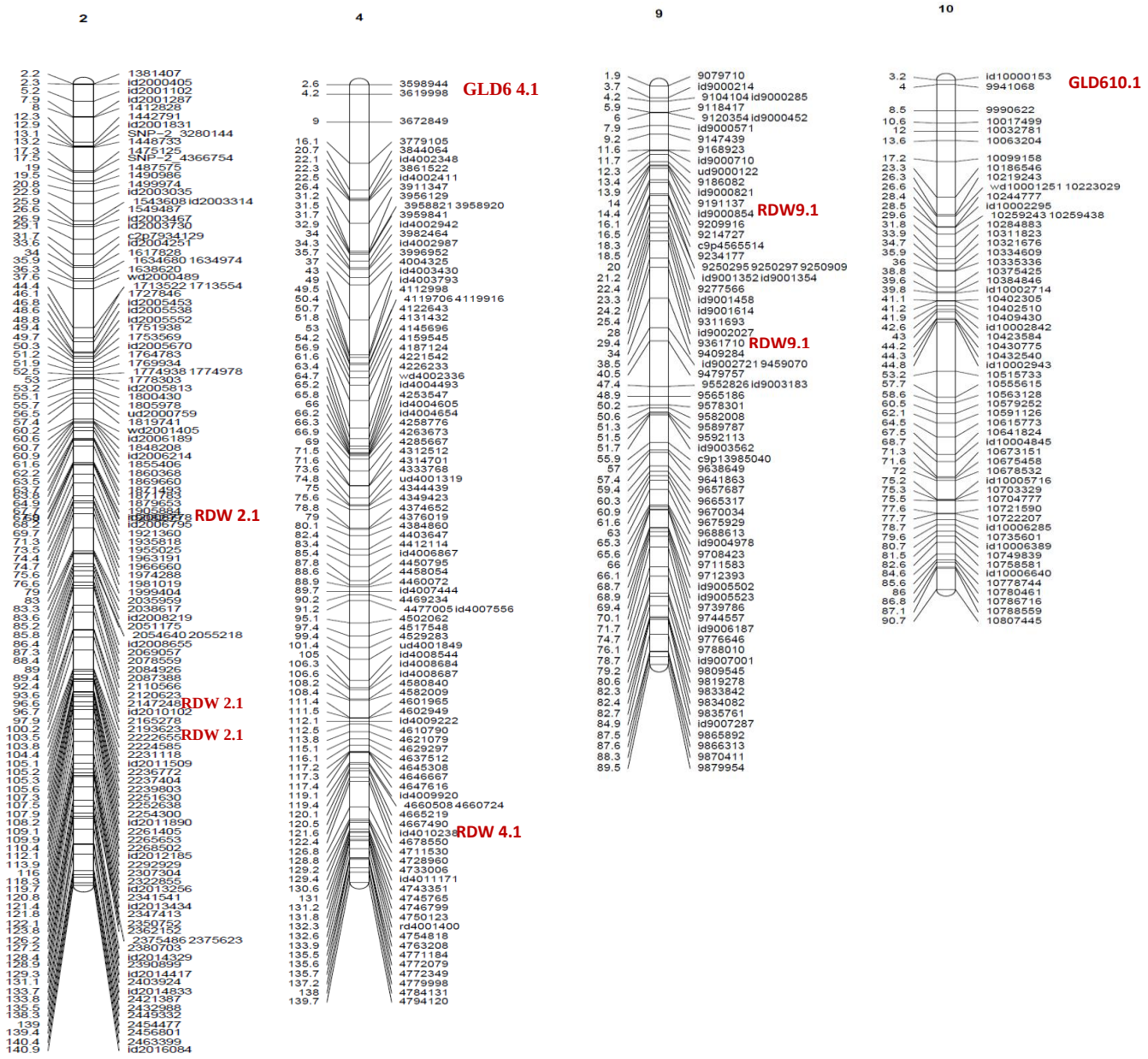


*Fig 20: Chromosomal location of QTLs identified from a population of 167 Supa / CK801 F2:3 using 1071 SNP markers and 16 iron toxicity tolerance traits measured at the seedling stage with a hydroponic system.*Significant QTLs (written in maroon) were identified for root dry weight (RDW) and green leaves at day six of toxic iron treatment

CHAPTER FIVE: DISCUSSION

5.1 Morphological and physiological responses of seventeen varieties

In this study, a clear morphological variation was observed among genotypesin response to toxic levels of iron which became pronounced with time of treatment. Stein *et al*. (2009) suggested a range of 30-150 ppm Fe^{2+} to be essential for plants metabolic processes therefore exceeding this limit usually shifts the cell redox balance toward a pro-oxidant state. The 300 ppm toxic Fe^{2+} concentrations used in this study enhanced production of visible morphological changes in genotypes one of the major one being leaf bronzing which became visible in leaves of some genotypes after four days of iron treatment. Thus we considered this character to be the major morphological signal of iron toxicity in rice seedling. The use of 300 ppm $FeSO_4$ for hydroponic screening of rice seedling for iron toxicity has been suggested (Venus *et al*., 2013).

High levels of iron added back to the solution in the first seven days of treatment positively correlated to the amount of phosphorus added back to the solution. This kind of increasing P/Fe^{2+} ratio in solution cultures was previously reported (Amberger and Mathan, 1977). However, based on phosphorus and Ferrozine control only approximately 3 ppm Fe^{2+} precipitated with P meaning the high amounts added were absorbed by the plant causing the visible leaf bronzing symptoms.

The low precipitation between P and Fe^{2+} in the solution culture was used to modify the Magnavaca solution used for screening rice for Aluminium toxicity to fit the nutrient requirements for screening rice for iron toxicity and to minimize the chemical interactions

between Fe and other mineral species in the nutrient solution at the high Fe concentrations(Famoso *et al.,* 2010). The high absorption of both phosphorus and Fe^{2+} would therefore suggest that the translocation of phosphorus was not interrupted in the presence of high iron levels and there was no internal tissues precipitation between phosphorus and Fe^{2+}. It is therefore worth noting that the essence of reduced phosphorus in the modified Magnavaca solution (Famoso *et al.,* 2010) is to make phosphorus available to plants and minimize any precipitation of this element in the solution culture.

Increased shoot length and lateral roots observed in the varieties with low leaf bronzing scores (3-5) is believed to be a morphological mechanism of these genotypes to ameliorate the harmful effects of toxic iron. Formation of such multiple new roots have been reported (Engel *et al.,* 2009) as a tolerance mechanism and could be used by rice under iron toxicity to continuously absorb nutrients from the solution. Other studies (Wu *et al*., 2012) linked lateral roots production to total radial oxygen loss and iron plaques formation thus suggesting that lateral roots contribute to reduction of the amount of iron entering the root. Dufey*et al*. (2009) reported that in severe cases of iron toxicity, rice plants showed reduction in root and shoot biomass which was in tandem with our observations in this study for all genotypes under Fetox with the sensitive (LBS 7-9) showing relative lower root: shoot biomass ratio compared to the tolerant genotypes (LBS 3). Root length was also reduced under Fetox across genotypes suggesting a possible decrease in root cell division and elongation. In different cases, excess iron have been reported to arrest primary root growth (Kang *et al.,* 2011; Li *et al*., 2015).

Chlorophyll pigments were low for the varieties with high leaf bronzing scores. This shows that leaf bronzing can be linked directly to disruption of photosynthetic machinery or it could possibly be the action of chlorophylase in the chloroplast to initiate the degradation of chlorophyll to chlorophylite (Noreen and Ashraf, 2009). Leaf bronzing could also be a result of accumulating high oxidized polyphenols which is often as a result of iron accumulation in the leaves (Yamauchi and Peng, 1993). Neves *et al*. (2009) reported impaired photosynthesis in leaves under high iron content which explains the reduced photosynthesis rate observed in this study under Fetox treatment. Stein *et al.* (2009) also observed reduced concentrations of chlorophyll a and b in susceptible cultivar and related it to photo-oxidation as a result of direct oxidative stress.

In other studies (Dingkuhn *et al.,* 1999; Awal and Ikeda, 2002; Sairam and Saxena, 2000), reduction in chlorophyll pigment has been reported to be in association with stomatal closure, leading to a reduction of photosynthesis. In this study, the same finding of decreasing conductance, chlorophyll pigments, and photosynthesis were observed under Fetox. However, these observations negatively correlate with the survival of plants since photosynthesis serves as a backbone of plant metabolism from which it derives its soluble sugars and in excess starch used for growth and other housekeeping processes. The reduction in photosynthesis observed in this study under Fetox is a negative feedback of a possible survival mechanism by varieties closing their stomata to reduce water loss through transpiration in order to reduce absorption of iron into the plants. As a result, the amount of CO_2 diffusing into the plant is also reduced thus resulting into reduced photosynthesis. According to Pereira *et al*. (2013), the reduction in leaf photosynthesis in rice under iron stress could be a result of oxidation of chlorophyll by free

hydroxyl radical originating from within Fenton reactions which in either case is still detrimental to plant survival under iron stress.

5.2 Physiological morphological and biochemical analyses of four varieties contrasting for iron toxicity tolerance

Plants respond to stresses by using recovery strategies that limit or correct the negative effects of the imposed stress. In this study, genotype response to toxic iron concentrations was evident in the manner that they actively altered their physiological and morphological attributes. This clearly elucidates these genotypes' intraspecific variability with respect to iron toxicity tolerance. Tolerant genotypes CK801 and Suakoko 8 significantly reduced their conductance in the second week of treatment as well as their transpiration rate. Becker and Asch (2005) explained that translocation of iron in the xylem follows the transpiration stream. Hence, stomatal closure is a mechanism of avoidance in order to limit the uptake and translocation of iron in the aerial parts. Similar results of low stomatal conductance under Fe toxic conditions were also reported by Dufey *et al.*(2009). Reducing the amount of water loss through transpiration, CK801 and Suakoko 8 were able to limit iron influx into the plant, in contrast to what happened in sensitive varieties IR64 and Supa. However, CK801 and Suakoko 8 recorded high CO_2 concentrations, which were directly correlated to the high photosynthesis rates observed in these genotypes. Reports show that limiting transpiration rate may affect CO_2 exchange rate and related photosynthetic functions, resulting in reduced plant growth (Goicoechea *et al*., 2001). This was not the case with the findings in this study which indicated that CK801 and Suakoko8 had good control of their stomatal opening, thereby efficiently balancing the water and gas fluxes through the stomata.

The high photosynthesis rates observed in tolerant genotypes also indicate that they did not suffer from photo-oxidation of the photosynthetic organelles. Besides, compared with sensitive varieties, these tolerant genotypes exhibited increased shoot length as well as more lateral roots. Moradi and Ismail (2007) suggested that reduction in transpiration rate could be one of the adaptive mechanisms used by rice to tolerate salinity. It is possible that reduced rates of transpiration and conductance, especially in the case of CK801, contributed to maintaining cell turgidity while diluting the effect of iron concentration. This school of thought can be supported by the fact that this genotype recorded the highest shoot biomass as well as the highest relative water content in its tissues. On the other hand, the high rate of photosynthesis seen in CK801 can also be related to high levels of soluble sugars accumulated in its shoots and high starch accumulated in its roots. These findings agree with those of Rout *et al*. (2014) who reported increased shoot growth in tolerant rice cultivars under iron-toxic environments. The sensitive genotypes recorded low photosynthetic rates and CO_2 concentrations, suggesting a possible degradation of the thylakoid membranes (Marschner, 1995) and oxidative damage within the chloroplast. Similar results were reported for rice under iron-toxic conditions (Stein *et al*., 2009; 2014).

According to Suh *et al*. (2002), excess iron in the plant gives rise to a significant increase in cytochrome b_6/f content of thylakoids, which results in a higher susceptibility of photosystem II to photo-inhibition and, consequently, lower photosynthetic rate and higher rate of singlet oxygen ($^1O_2$) production. This could be a possible explanation for the high leaf bronzing score and high MDA levels recorded for these sensitive genotypes. Several other factors, including the accumulation of high starch and soluble sugars noted in sensitive genotype Supa can therefore be

explained by the poor adaptation of this genotype to Fe toxicity, which translates into inefficient utilization of carbon reserves for its physiological and metabolic processes. Supa also had low relative water content in the leaves, which could have led to the loss of guard cell turgor and thus to reduction in stomatal conductance. Supa also showed increasing chlorophyll fluorescence with stress duration, which could be related to the high dissipation of internal energy generated from the excitation of electrons as a result of damage caused by Fe toxicity on the photosynthetic apparatus. This could result in ROS production from photosystem II as well as increased heat dissipation, causing photo-oxidative damage to the photosynthetic apparatus and thereby reducing photosynthesis. Similar findings have been reported for sorghum (Netondo *et al*., 2004). In other studies, Suh *et al.* (2002) showed that iron excess led to photo-damage of photosystem II, derived from excessive production of singlet oxygen in pea plants, while, Kampfenkel *et al.* (1995) reported that excessive amounts of iron led to photo-inhibition, increased reduction of photosystem II, and higher thylakoid energization in *Nicotiana plumbaginifolia* cuttings exposed to excess iron.

In all genotypes, a reduction in root length was observed, confirming root growth inhibition as one of the negative effects of iron toxicity (Li *et al.,* 2015). Other reports show that roots are affected by iron toxicity and usually become short, coarse, blunted, and dark brown in color (Genon *et al.,* 1994). However, in this study, the tolerant genotypes formed new roots despite the stress as opposed to the sensitive genotypes. This was viewed as a mechanism used by the tolerant genotypes to enable them to continuously absorb nutrients to support growth and avoid the possible effects brought about by nutrient precipitation on the active root nutrient uptake sites. Kang *et al*. (2011) showed the formation of lateral roots in tolerant NERICA sister lines

and suggested that it could be closely associated with reducing iron absorption. A similar phenomenon was also reported in rice varieties under iron toxicity tolerance screening (Engel *et al*., 2009).

In all genotypes, there was a remarkable variation in chlorophyll a and b contents, with the tolerant genotypes maintaining high levels of both pigments. The chlorophyll pigment of these four genotypes was directly proportional to their leaf bronzing scores and photosynthesis rates. The low level of chlorophyll pigments observed in the sensitive genotypes could be as a result of low metabolism status observed in Supa under Fe toxicity; damaged roots resulting in poor nutrient absorption; or impairment of CO_2 fixation in the chloroplast due to photo-oxidation, which might have resulted from higher thylakoid energy from the photosystem II (Asada, 2006). This kind of inefficient energy transfer in the photosystems can lead to the production of ROS as a result of electron leakage from the Fe-S centers of photosystem I and reduced ferredoxin (Mittler *et al.,* 2004; Gechev *et al*., 2006). Several studies have also reported reduction in chlorophyll pigment as being associated with stomatal closure, leading to reduced photosynthesis (Dingkuhn *et al*., 1999; Awal and Ikeda, 2002; Sairam and Saxena, 2000) as was observed among sensitive genotypes.

5.2.1 Starch and soluble sugars

Starch is a major storage carbohydrate in plants. It fuels plant metabolism and growth when plants are unable to photosynthesize at night in the absence of light (Smith and Stitt, 2007; Sulpice *et al*., 2009). Plants tend to store starch in their leaves for the short term and in roots and seeds over long periods. Hence, starch stored in the leaf plays a pivotal role in the plant's daily carbohydrate metabolism (Gibon, 2004; Streb and Zeeman, 2012). In this study, starch content was higher in the iron-treated seedlings than in the control; higher amounts were recorded in the

shoots compared with those in the roots and, particularly, in sensitive genotype IR64. These findings show that high starch levels resulting from either an increased rate of starch synthesis or a decreased rate of starch breakdown could be a general response of rice to Fe toxicity independently of tolerance level. Whether this is a way of anticipating starvation because Fe toxicity deeply affects photosynthesis or the result of the overall reduction of plant metabolism under Fe toxicity needs further investigation.

The high starch content observed in iron-treated plants was consistent with the decreasing levels of soluble sugars in the same genotypes tested in this study. According to Couée*et al*. (2006), soluble sugars can be involved in, or related to, ROS-producing metabolic pathways, but they can also feed NADPH-producing metabolic pathways, such as the oxidative pentose-phosphate (OPP) pathway, which can contribute to ROS scavenging. Thus it is tempting to suppose that rice maintains a low level of soluble sugars to control ROS production, but this can also make the plants vulnerable because soluble sugars are important sources of carbon and energy that support growth. Low soluble sugar levels could also result from reduced photosynthesis.

The high levels of starch recorded for IR64 in both root and shoot can be attributed to the fact that it invested more in starch at the expense of its growth compared with tolerant CK801, which had low starch in its shoot with high growth. Varying starch accumulation in the genotypes at different sampling points indicate the transitory nature of starch occurring at different developmental times. Hence, it could serve to temporarily increase local sink strength to supply carbohydrates during periods of carbon hunger for sustained growth and metabolic processes (Pantin *et al*., 2011; Geiger *et al*., 2000). Some studies have reported that soluble sugar

fluctuations under abiotic stresses involve changes in CO_2 assimilation and source-sink carbon partitioning (Roitsch, 1999; Gupta and Kaur, 2005).

Tolerant genotypes CK801 and Suakoko 8 were observed to accumulate high starch in the shoots and roots, respectively, while maintaining high biomass. This positive correlation between starch and biomass indicates that these two genotypes were able to maximize growth with increasing starch reserves, thus balancing their carbon supply and their growth. The sensitive genotype Supa recorded increased starch content as well as increased soluble sugar under Fe-toxic conditions. This shows that the genotype immobilized its starch reserves and sugars, and this could be attributed to the compromised growth observed in this genotype, implying that assimilated carbon, which could be used to produce new photosynthetic biomass, languishes as an unproductive storage compound. However, the premature exhaustion of carbohydrate reserves observed in tolerant genotype CK801 at sampling point 2 in the shoots could also be detrimental if this is not supplemented by reserves in the roots as it may trigger a starvation response in which valuable components of the cells (such as proteins) are degraded to support cellular housekeeping activities to keep the plant alive (Yu, 1999).

5.2.2 Lipid peroxidation and membrane stability

Increasing MDA levels observed in sensitive genotypes Supa and IR64 across sampling points can be linked to increased level of lipid peroxidation across the screening period. Enhancedlipidperoxidationunder irontoxicityhasbeen reportedfordifferentspecies (Souza-Santos*etal*., 2001; SinháandSaxena, 2006; Kuki*etal*., 2008; Kumar*etal.*, 2008). According to Xing*etal*. (2010), intensity ofoxidative damage differs among genotypes. This observation is

further supported by the membrane stability finding in this study which shows high cell injuries in sensitive genotypes as opposed to those seen in tolerant genotypes CK801 and Suakoko 8.

The steady increase in the levels of MDA from one sampling point to another was directly proportional to the occurrence of leaf bronzing symptoms observed during the growth of the genotypes in the iron toxic nutrient solution. This can thus be an indicator of progressive disruption of membranes, metabolic processes, anatomical alterations, and obstructions of photosynthesis. Our findings are therefore in tandem with those reported for iron-induced lipid peroxidation in rice leaves (Kao *et al.,* 2001; Stein *et al*., 2008). For tolerant genotype CK801, despite high levels of iron concentrations recorded in the upper leaves, MDA was maintained at relatively low levels throughout the screening period, suggesting that strong mechanisms to counteract the harmful effects of iron were at work. Similarly, high iron tissue accumulation with low MDA levels have also been reported in rice (Muller *et al*., 2015). This we can also relate to the low leaf bronzing score of 3 recorded for this genotype.

5.2.3 Tissue iron and leaf bronzing

The analysis of iron concentration in the upper four leaves and the lower three leaves and total root iron from all genotypes indicated high accumulation of upper leaf iron, especially in sensitive genotype Supa. Thus, the high leaf bronzing score for Supa could have resulted from accumulation of high levels of iron in the shoots. Similar findings were reported by Silveira *et al.* (2007) and Stein *et al.* (2009) who while screening rice for iron toxicity tolerance observed similar pattern of high tissue iron for sensitive cultivars. The tolerant genotype CK801 recorded higher levels of upper leaf and root iron compared with sensitive IR64 but lower leaf bronzing

score (3 vs 7 for IR64). Such a pattern indicates the ability of CK801 to successfully deal with high amounts of intracellular iron and maintain growth under Fe stress. Pereira *et al*. (2014) and Wu *et al*. (2014) reported high shoot iron accumulation in tolerant rice varieties tested in their studies using iron sulfate in more or less similar concentrations as those used in this study.

In several other studies, mechanisms of leaf tissue tolerance for high levels of iron have been suggested as important features that rice cultivars use to tolerate high levels of iron in the shoots (Wu *et al*., 1998; Asch *et al*., 2005; Stein *et al.*, 2009, Engel *et al.*, 2009). The low tissue iron in both roots and shoots recorded for tolerant genotype Suakoko 8 can be attributed to the exclusion of ferrous at the root level. This can be achieved by using the roots' oxidizing power, thereby precipitating ferrous into ferric oxides and avoiding a high influx of this toxic form of iron into the plant tissues. Engel *et al*. (2009) reported the same finding for tolerant rice cultivars under iron toxicity tolerance screening. However, Suakoko 8 was also observed to contain iron in the lower and upper leaves, explaining its leaf bronzing score of 5. Similar results on root oxidizing power were reported by Engel *et al*. (2012) and Wijerathna *et al*. (2012) for rice as a mechanism for tolerating iron-toxic environments. In the same manner, tissue iron partitioning in the lower leaves was also reported in rice (Majerus *et al*., 2007b, Stein *et al.*, 2009; Engel *et al.*, 2012), thus explaining the observed lower shoot iron concentrations in all the genotypes used in this study. However, this was not exploited as a major tolerance mechanism in either of the tolerant genotypes as only little amounts of iron were deposited in these tissues.

5.2.4 Anti-oxidant and anti-oxidative responses

The activities of anti-oxidant enzymes obtained from this study vividly express the difference in responses among the four genotypes to iron-toxic environment. Immediately after the seedlings were subjected to toxic iron concentrations, all genotypes strongly exhibited divergent responses to the stress imposed, by either actively increasing or reducing the activities of anti-oxidative enzymes. Tolerant CK801 was identified to have maintained relatively high levels of SOD and GR in the shoots and roots as well as high levels of APX and CAT in the roots. This indicates that this genotype has a very good strategy of scavenging for ROS due to the fact that SOD can scavenge for ROS in all cellular compartments and act to reduce the highly toxic superoxide to the less toxic H_2O_2 (Asada, 1999; Mittler, 2002; Moradi and Ismail, 2007; Stein *et al*., 2014). Elevated levels of APX and CAT in the roots and of POX in the shoots of CK801 can therefore be linked to an efficient H_2O_2-scavenging mechanism, thus decreasing their chances of accumulating in plant tissues. Significantly high levels of GR maintained in the shoots and roots also clearly elucidate the strengthening of ROS detoxification systems in this genotype and corresponds to increasing levels of TPC in this genotype across time. These results confirm the findings of Stein *et al.* (2014) who also observed increased GR, CAT, and SOD activities in tolerant rice genotypes grown under iron-toxic environments. According to Rao *et al*. (2008), GR is a potential enzyme of the ASC–GSH cycle, which plays an essential role in the plant's defense against ROS by maintaining the reduced glutathione (GSH) level. Hence, we can confidently say that CK801 has a strong ROS-scavenging system that acts promptly to mitigate the harmful effects of ROS and that confers its observed tolerance for iron toxicity.

Suakoko 8 was observed to increase its SOD activity at sampling points 3 to 5 with a concomitant increase of CAT activities at the same sampling point; but it showed decreasing APX activities across sampling points. Reduced APX activity in the roots and shoots indicates poor efficiency in decomposing H_2O_2 in the roots because, according to Asada (2006), APX plays a vital role in plant defense against oxidative stress by scavenging H_2O_2 in chloroplasts, cytosol, mitochondria, and peroxisomes of plant cells. However, in this genotype, CAT activity increased and this enzyme is assumed to play a pivotal role in detoxifying the H_2O_2 produced and so were the elevated levels of POX activity in both roots and shoots. These observations are in agreement with those of Saikia and Baruah (2012) who reported increased SOD and APX activities in rice under iron toxicity stress. Similar findings of increased SOD and POX were also noted (Bode *et al*., 1995) for rice under iron toxicity.

SOD activities were observed to be decreasing in sensitive genotypes Supa and IR64 immediately after the application of toxic iron. This was followed by decreasing activities of APX and CAT in the shoots of these two varieties, but with IR64 having elevated levels of APX and CAT activity in the roots and with Supa maintaining relatively high CAT activities in the roots. The declining SOD, CAT, and APX activities observed in the shoots after application of toxic iron concentrations could have compromised the protection of these genotypes against superoxide and H_2O_2 and, as such, may favor over accumulation of superoxide and H_2O_2, leading to high lipid peroxidation. The decrease in SOD activities under excess Fe has also been reported (Fang *et al*., 2001) in leaves of *O. sativa* under an iron-toxic environment. However, in contrast to our findings, Stein *et al*. (2009) reported increased SOD activity in sensitive rice cultivars grown in iron-toxic areas and this was attributed to the consequences of oxidative stress

generated by the higher amounts of iron in the low-molecular-mass fractions, rather than to a mechanism used by the sensitive cultivar to achieve tolerance for iron excess. For these two genotypes, therefore, a quick remedy is needed to correct the weakened superoxide- and H_2O_2-scavenging systems if they are to survive the adverse effects of Fe toxicity. In line with this, IR64 was observed to increase its POX activity at sampling points 3 and 4 but it sharply declined at sampling point 5, indicating either exhaustion or inhibition of new enzyme synthesis (Mittler, 2002). However, we cannot overrule the possibility that the increased POX activity was a positive response mechanism by the genotype in trying to detoxify the H_2O_2 accumulating in its tissues.

5.3 Histochemical analysis

In the recent past, researchers have focused their studies in radial oxygen loss as a mechanism used by rice to tolerate anoxic environments (Visser *et al*., 2000; Colmer, 2003b; De Simone *et al.*, 2003; Armstrong and Armstrong, 2005; Soukup *et al.*, 2007;Garthwaite *et al*., 2008; Kotula *et al*., 2009; Shiono *et al.*, 2011; Shiono *et al.*, 2014). Suberin and lignin deposition in both the stem and roots is suggested to play vital roles in acting as a barrier preventing radial oxygen loss across the stem and root base (Watanabe *et al.*, 2013; Shiono *et al*., 2014). In our study, we observed suberin and lignin deposition in the roots and stem base of all the four genotypes with varying density from genotype to genotype. According to Franke and Schreiber (2007), apoplastic barriers in endodermal and hypodermal cell walls of roots contain varying amounts of suberin, lignin, and carbohydrates, a confirmation of our findings.

In this study, suberin and lignin was evaluated based on the intensity of histochemical stain used. Suakoko 8 was observed to have high lignin and suberin deposition from the stem down to the

root cap both in the inner and outer parts of the roots; the same was true for CK801 but with reduced inner lignin and suberin deposition in the inner root cap. Several researchers (van de Mortel *et al.*, 2006; Kotula *et al*., 2009; Krishnamurthy *et al.*, 2009; Ranathunge *et al*., 2011) have reported that both suberin and lignin localize at the inner part of the roots (endodermis) and also in the outer parts of the root (sclerenchyma and exodermis) and both function as a barrier that impedes the radial diffusion of oxygen to the rhizosphere. Insalud *et al*. (2006) and Kotula *et al*. (2009) observed that the pattern of suberin and/or lignin accumulations in the roots matched the localization of the ROL barrier. In our study, the observation of differential suberin and lignin accumulation in the genotypes tested can be related to different ways by which the genotypes used in this study transfer oxygen from the upper parts of the shoot to their roots as was evident for Suakoko 8 and Supa.

The suberin and lignin patterns observed in the sensitive genotype Supa would suggest that it is able to direct the diffused oxygen from the upper shoots down to the roots, but with a possible loss across the root pores immediately after the root base. In this case, the concentration of oxygen that reaches the root tip may not be sufficiently high to effectively oxidize ferrous into the ferric form. Li *et al*. (2015) have shown that root growth inhibition under Fe-toxic condition occurs only when the root tip is in contact with toxic levels of iron. It is therefore critical to keep the root tip free from ferrous to pursue growth. On the other hand, it is well known that suberin and lignin play a crucial role in solute transport through apoplastic barriers (Franke and Schreiber, 2007). It is thus possible that genotypes with weak suberization/lignification of their cell walls are not able to selectively permeate the solutes being absorbed at the root cap, thereby allowing excess influx of toxic iron into the plant tissues. The high accumulation of suberin and

lignin observed in the inner and outer root of Suakoko 8 confirms the excluder character of this genotype. Jackson and Armstrong (1999), Deng *et al*. (2009), and Wu *et al*. (2012) also reported that internal O_2 diffusion in roots of rice with some leakage to the rhizosphere, particularly around the tip of each adventitious root and from lateral roots, plays a role in protecting the plant from toxic compounds such as Fe^{2+}, Mn^{2+}, As, Pb, Zn, and H_2S. Based on this, it was concluded that the tolerance for toxic iron levels observed in Suakoko 8 is related to its high root oxidizing power. Similar findings of root oxidation have been reported (Ranathunge *et al*., 2011a; Abiko *et al*., 2012). Studies by Shiono *et al*. (2014) have also identified suberin as a major constituent of the radial oxygen loss barrier in roots of rice. Hence, in the other three genotypes, the decreasing suberin and lignin accumulation toward the root tip could have possibly hindered or minimized oxygen accumulation at the rhizosphere. Based on the findings in this study, a diagrammatic illustration was developed of a possible oxygen sequestration from the shoots to the roots in tolerant and sensitive genotypes used in this study as below (Fig.21).

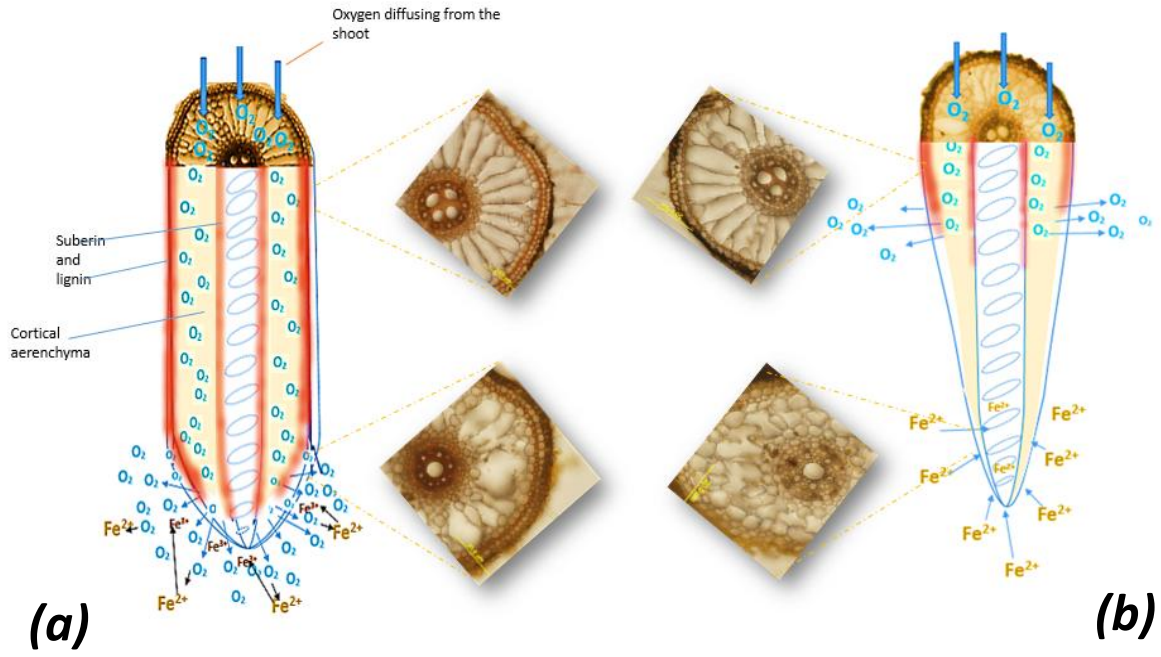


Fig 21: Illustration of aerenchyma development, suberization, and lignification in the roots of tolerant (a) and sensitive (b) rice genotypes.

5.4 Gene expression analysis of two rice genotypes

Differences in gene expression was apparent between the two test varieties, tissues and time. Supa which is a sensitive variety majorly employing inclusion and tissue tolerance but with subsequent collapse after day 10 of excess Fe treatment recorded the highest number of upregulated genes at day 7 of iron treatment in the root. Significant upregulation of most genes involved in celullar metabolism was noted across tissues and days for CK801 which has been identified as a true tolerant variety employing both avoidance of excess Fe at the root with concomitant tissue Fe tolerance over time (early study unpublished). This clear difference in gene expression between these two varieties signifies a possible difference in adaptation mechanism by which they use to combat the harmful effect of excess Fe in their metabolic pathway. Just like in the studies (Quinet *et al*., 2012; Wu *et al.*, 2016) of rice subjected to chronic and acute Fe stress respectively, more genes were positively expressed in roots than in shoots across the two days of Fe treatment in each case.

A remarkable down regulation of all genes involved in DMA biosynthesis in the roots of both varieties except *OsSAMS2* and *OsYSL15* at day 3 of CK801 as well as transporters and related genes for ferrous Fe uptake/translocation of Fe treatment could show that at this stage of growth the plants were still utilizing Fe in the form of Fe^{2+} maintained under steady balance (control) or it could be a possible early down play by these varieties upon sensing presence of excess Fe^{2+} in their growth environment as they try to reconfigure their metabolic framework. This kind of down regulation of genes involved in Fe^{3+} DMA transport under Fe toxic conditions have been

proposed (Onaga *et al*., 2016) thus in agreement with our finding however, at day 7, all the genes involved in DMA biosynthesis except *OsSAMS2* in both the varieties was upregulated suggesting the presence of Fe^{3+} at the rhizosphere which could be as a result of oxidation of the culture media or from radial oxygen loss at the root of these varieties. This is supported by a further upregulation of ferric reductase genes *OsFRO1* and *OsFRO2* which are then sustained by upregulation of ferrous Fe transporters *OsIRT1* and *OsNRAMP*1 in the same roots. Studies (Ishimaru *et al*., 2006; 2012) reports that Fe^{2+} uptake from the rhizosphere is predominantly mediated by *OsIRT1and OsNRAMP*1 thus a clear indication that at day 7, high Fe^{2+} was being absorbed by the both varieties as opposed to day 3. The upregulation of genes involved in Fe^{2+}uptake observed in this study is in harmony with those reported in early studies (Bashir *et al*., 2014; Finatto *et al*., 2015).

The upregulation of Fe transporters of the yellow strip like family *OsYSL2* in the roots of both variety at day 7 also supports the translocation of Fe^{2+} with a higher upregulation observed in Supa compared to CK801. *OsYSL18* which is involved in Fe distribution in the plant is positively expressed in both varieties with a high upregulation in Supa indicating the presence of high internal root tissue Fe in this variety which is further confirmed with tissue %Fe analysis (Table 11b). Regardless of down regulation of most ferrous and ferric uptake gene at day 3 of the treatment, *OsVIT2* involved in Fe transporter into vacuole and sequestration was upregulated in all varieties and tissues at day 3 and day 7 except in the shoot of CK801 at day 7. This observation shows the presence of Fe in the tissues of these varieties with higher ration on Supa in all tissues in all days which can be regarded as negative mechanism in the effort to survive in a Fe toxic environment however, the low upregulation ratios in CK801 in both tissues and the

down regulation in the shoot at day 7 indicate a possible low amounts of Fe in the upper shoot tissues which is further expressed in the number of dry leaves which is brought about by bronzing resulting from oxidative damage of the leaf membranes as a result of excess Fe^{2+} in the leaf tissues. This was further supported by the reduced %Fe in the shoots of CK801 (Table 11b). Fe transportation into the mitochondria was evident with the upregulation of *OsMIT* and *OsMIR* and into the chloroplast by *TIC21* with a simultaneous compartmentalization in the ferritin by *OsFer1* and *OsFer2* in both varieties. Transcriptional regulator genes *OsIRO2, OsIRO3* and *OsbHLH133* were all down regulated in the roots of both varieties at day 3 and upregulated in the roots at day 7 suggesting high influx of Fe^{2+} at day 7. A strong down regulation of the same transcriptional factors was recorded in the shoot of CK801 at day 7. This kind of differing regulation of transporters and chelators for subcellular iron storage was reported (Bashir *et al*., 2014; Onaga *et al.*, 2016; Quinet *et al*., 2012)

Most of the genes involved in ROS detoxification were upregulated in both varieties and tissues across the two days with an exception in the shoot of Supa at day 7 where only few genes were upregulated. This positive expression which started as early as day 3 would suggest good defence mechanism in scavenging and detoxification of ROS by both varieties upon sensing excess Fe^{2+} in their environment. Similar findings of upregulation of genes involved in ROS detoxification has been reported in rice (Quinet *et al*., 2012; Bashir *et al.*, 2014, Wu *et al*., 2016). However, reduced upregulation of these genes in the shoots of Supa at day 7 of Fe treatment would suggest a reduction or inhibition of signal transduction pathway in this variety with increasing time of Fe toxicity thus a possible explanation of the collapse observed in this variety at day 10 of Fe treatment. Conversely, increased upregulation of these detoxification genes

observed in CK801 would suggest a good strategy employed by this variety in sending accurate signals of switching on and off genes involved in the defence pathway thus a confirmation of increased activity of anti-oxidative enzymes and anti-oxidants observed in this variety under Fe toxic condition.

In tandem with the proposed Fe nutrition (Onaga *et al*., 2016) for includers and excluders, where genes such as *OsIRT1, TOM1, OsYSL15, OsDEF1, OsIRO2, OsNRAMP1, OsNAAT1, OsNAS1, OsDMAS1* were hypothesized to be downregulated under Fe toxicity, In this study all these genes were found to be down regulated at day 3 of Fe treatment with progressive upregulated in the roots of both varieties thus in opposing this hypothesis. However, other genes which were hypothesized (Onaga *et al*., 2016) to be upregulated in roots of both excluders and includers such as *OsIRO3, OsbHLH133* were also found to be upregulated in the two test varieties hence confirms their hypothesis. Genes such as *OsNAAT1* which have been reported (Finatto *et al.,* 2015) to be induced under Fe toxicity in shoot was as well in agreement with our findings where such a gene was up regulated in the shoots of both varieties in our study and down regulated in the root of both variety at day 3 of Fe treatment.

5.5 QTL mapping

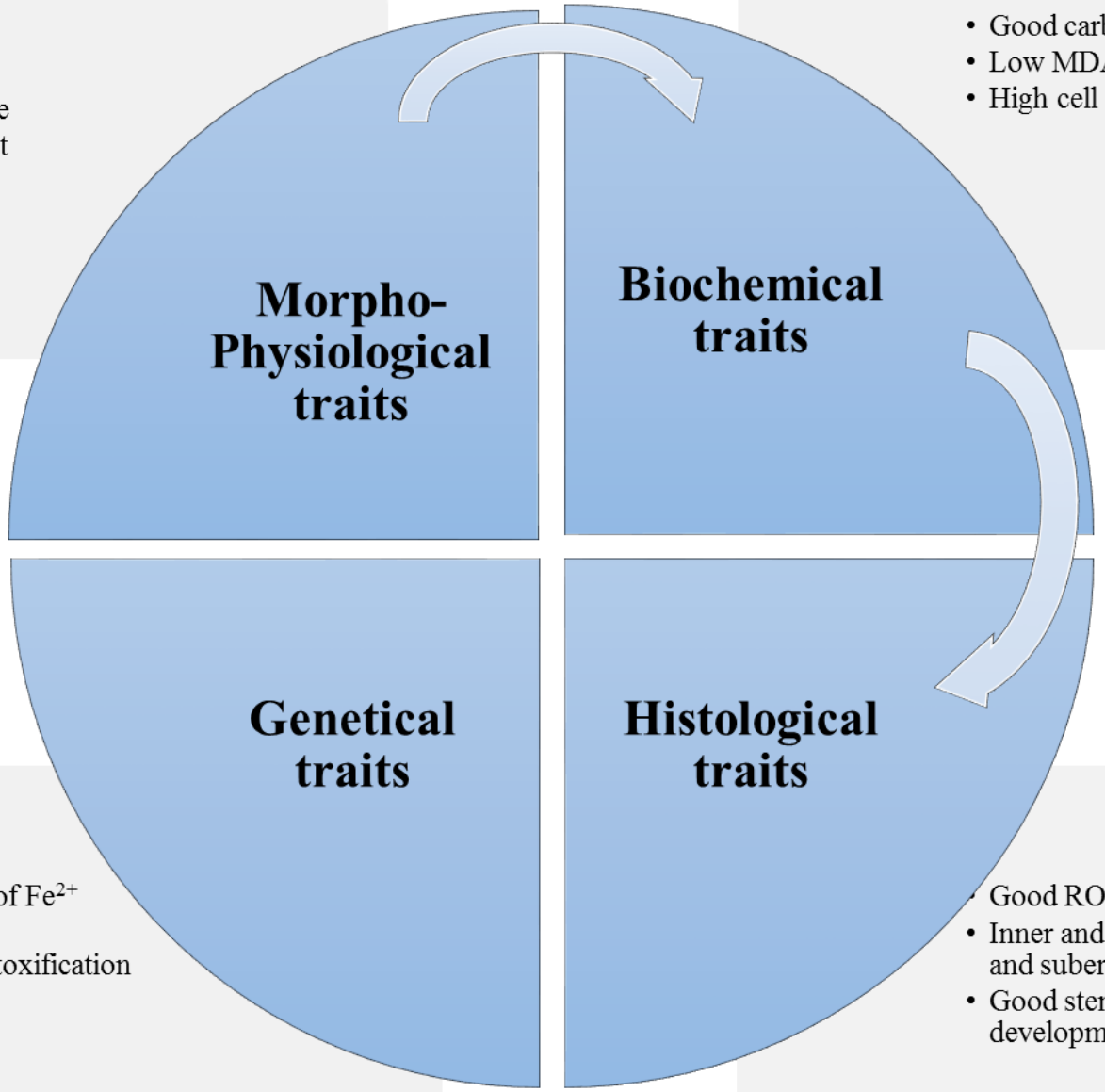
A number of mapping studies have identified QTLs associated with iron toxicity tolerance in rice Shimizu *et al*., 2005; Wu *et al*. 1998; Wan *et al*., 2005; Dufey *et al*., 2009, 2012; Fukuda *et al*., 2012; Quinet *et al*., 2012 Bashir *et al*., 2014; Dufey *et al*., 2015*b*). Several genetic studies reflect that iron toxicity tolerance is a complex quantitative trait controlled by a large number of rather small effect QTL (Fukuda *et al*., 2012; Wu *et al*., 2014; Dufey *et al*., 2015*a*, 2015*b*), affirming

the involvement of multiple tolerance mechanisms. Thus, manipulation of a single or a few of these QTL might not contribute to strong phenotypic modifications. Evidently more efforts are needed to identify new donors and alleles of stronger effect. Few studies detected common QTL using different donors, whereas in other studies, QTL for different traits associated with iron toxicity responses co-localise in the same region. For instance, Wu *et al*. (1997) identified QTL for leaf bronzing and shoot dry weight on chromosome 1 and 8, explaining 10–32% of the phenotypic variation. It is noted that in the same region, QTL associated with enzymatic activity of antioxidants in rice leaves were detected (Wu *et al*., 1998). Similarly, Fukuda *et al*. (2012) detected a region on chromosome 3 (between R663 and S1571) responsible for high shoot iron content in a susceptible variety, which co-localise with the QTL previously identified by Shimizu *et al*. (2005) for the same trait. While evaluating the same mapping population in different experimental systems, Dufey *et al*. (2012a) identified six regions of the rice genome where detected QTL are consistent over different experimental conditions, different environments and even between independent mapping studies. In a study of chronic versus pulse iron toxicity stress, Wu *et al*. (2014) identified several QTLthat co-localise with or were close to QTLdetected in several other studies (Wu *et al*., 1998; Wan *et al*., 2005; Dufey *et al*., 2009, 2012b; Fukuda *et al*., 2012). Recently, Dufey *et al*. (2015*b*) used an interspecific population of *O. sativaO. glaberrima* and identified several regions that were reported previously. Colocalisation of most of these QTL was captured in an integrative genetic map including 14 of the mapping studies conducted in different conditions of Fe toxicity (Dufey *et al*., 2015*a*). Inourstudy, QTL forrootdryweightwerefoundco-localizedinchromosomes 2, 4 and 9. ThisiscontrarytothefindingsofDufey*etal*., 2015b whowhileconductingan integrative map of 14 studies using different populationsfound the QTL for root dry weight in chromosomes 1 and 3.

QTL for green leaves identified in this study in chromosomes 4 and 10 could be used to enable dissection of the genetic control of iron toxicity tolerance mechanismsinceleafbronzingisthebasicindicatorforiron toxicity inrice. Thiscan thus open up the possibility of future efforts to develop varieties with improved iron toxicity tolerance by precisely transferring this QTLs into popular varieties and pyramiding multiple relevant QTLs for a particular iron toxic-prone environment. Alsointhis study QTLs were identified through composite interval mapping (CIM) using QGENE program. CIM has long been identified to have some advantages over other mapping methods in identifying smaller QTLs that may otherwise be masked by other larger QTLs. Zeng (1994) suggested some advantages of CIM over other mapping methods in three ways; (1) by confining the test to one region at a time it reduces multiple dimensional search problem - for multiple QTLs- to a one dimensional search problem (2) by conditioning linked markers in the test, the sensitivity of the test statistics to the position of individual QTLs is increased, and the precision of QTL mapping can be improved; and (3) by selectively and simultaneously using other markers in the analysis, the efficiency of QTL mapping can also be improved.

5.6 Summary for breeding superior iron toxicity tolerant varieties

- New green leaves
- Functional lateral roots
- Low leaf bronzing
- Good photosynthesis rate
- High chlorophyll content
- Low fluorescence



- Good ROS activity
- Good carbon assimilation
- Low MDA/ Membrane stability
- High cell water retention

- Up and down regulation of Fe^{2+} transporter genes
- Up regulation of ROS detoxification genes

- Good ROL function
- Inner and outer stele lignification and suberization
- Good stem and root aerenchyma development

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

6.1.1 Overall conclusion

It is clear from this study that the morphological traits alteration observed as a result of induced iron stress is a subset of plants' readjustment to its physiological responses under stress. Leaf bronzing is an evident symptomatic response to iron toxicity resulting from degradation of photosynthetic pigments which in turn leads to reduced photosynthesis. Furthermore, photosynthetic capability is aggravated by stomatal closure in an attempt to reduce transpiration rate, resulting to decreased CO_2 accumulation. Since plant growth majorly depends on the soluble sugars which is a product of photosynthesis, reduced photosynthesis therefore go a long way in depleting starch reserves which is a carbon store that fuels plant metabolism and growth. As a matter of fact, the damage imposed on plant photosynthetic pigments is the genesis of the crackdown in all the heterotrophic life process. On the other hand, it was established that gene response for the two test varieties (CK801 and Supa) to Fe toxicity is differentially regulated. Most genes in the two test varieties were upregulated in the roots at day 7 of Fe treatment as opposed to day 3 of Fe treatment where most genes were down regulated. Fe toxicity tolerance genes and molecular markers useful in breeding has been identified and now available for use in breeding.

6.1.2 Output and Outcome

1. This study have singled out key physiological and morphological traits including leaf bronzing, shoot length, lateral root, chlorophyll contents and photosynthesis rate as strong indicators linked to iron toxicity tolerance studies.

2. CK801 has been identified to be a true tolerant genotype using various mechanisms ranging from dilution effect (by maintaining high water content in its tissues and growing tall, thus producing new tissues) to having a very strong ROS-scavenging system in both roots and shoots as well as making good use of ROL to reduce ferrous back to ferric at the rhizosphere.

3. Suakoko 8 has been identified to explore root oxidizing power as a major tolerance mechanism with relatively good ROS-scavenging system both in the roots and shoots. Supa can be characterized as sensitive based on its poor performance under Fe toxicity as evidenced by the physiological and biochemical analyses conducted in this study. On the other hand, IR64 can be characterized as moderately tolerant to Fe toxicity based on its fluctuating responses to various physiological and biochemical activities tested in this study.

4. Supa which is a sensitive includer, positively expressed several genes both involved in Fe^{2+} uptake, homeostasis and ROS detoxification in its roots with increasing number of day of excess Fe treatment with decreasing upregulation in the shoots at day 7 of Fe treatment suggesting the presence of elevated levels of Fe in its shoots increases with time which can further lead to increased generation of ROS leading to membrane damage observed as high scores of leaf bronzing in this variety.

5. Observed down regulation of most genes in CK801 can be generalized as a positive adaptive mechanism in which this variety upon realization of Fe toxic environment moderates the signal transduction pathway by switching on and off of the genes involved in Fe uptake and translocation to regulate the amount of Fe going into its tissues thus avoiding the metabolic shift to a pro-oxidant state thereby sustaining its growth in an environment with elevated levels of Fe^{2+}.

6. It is therefore worth noting that apart from the use of root oxidizing power, tissue Fe dilution, ROS scavenging by anti-oxidative enzymes and antioxidants identified to be used by CK801 as a tolerance mechanism, down regulation of most genes involved in Fe nutrition in both early and late days of exposure to excess Fe is a strong molecular mechanism used by this variety to tolerate Fe toxicity.

7. QTL for root dry weight has been identified in chromosomes 1 and 3 while QTL for green leaves has been identified in chromosomes 4 and 10. This finding could be used to enable dissection of the genetic control of iron toxicity tolerance mechanismsinceleafbronzingisthebasicindicatorforiron toxicity inrice.

8. Finally, one variety (CK801) from this study has been identified as a true tolerant to iron toxicity and has been used as a recurring parent in developing iron tolerant population in a cross between CK801 and Supa.

6.2 Recommendations and future research

6.2.1 Recommendations

1. The use of modified Magnavaca nutrient culture for iron toxicity screening is endorsed as the most optimized way of maintaining uniform Fe^{2+} concentration in solution cultures throughout the screening period.

2. The cultivation of the newly developed rice hybrid (CK801 x Supa) is highly recommended in areas prone to iron toxicity.

6.2.2 Recommendations for future research

1. Even though this study has generated knowledge on the physio-chemical mechanisms underlying iron toxicity tolerance of some genotypes used in this study, an exploration of metabolites associated with iron toxicity tolerance in the same genotypes would increase the strength of correlations between physio-chemical and metabolic processes identified. Therefore, metabolome profiling is suggested as this will further provide a richer source of information about signaling and other post-transcriptional processes involved in toxic iron tolerance mechanisms.

2. Effort should be made by breeders to improve yield of the hybrid developed in this study by pyramiding so as to kill two birds “iron tolerance and yield” with one stone.

REFERENCES

Abiko, T., Kotula, L., Shiono, K., Malik, A. I., Colmer, T. D. and Nakazono, M. (2012). Enhanced formation of aerenchyma and induction of a barrier to radial oxygen loss in adventitious roots of *Zea nicaraguensis* contribute to its waterlogging tolerance as compared with maize (*Zea mays* ssp. *mays*). *Plant, Cell and Environ.* 35:1618–1630.

AfricaRice Annual report (2008). Responding to the rice crisis. Cotonou, Benin: 60pp

Agricultural Economic Review (2008). National Irrigation Board Strategic Plan 2008 – 2013 and Vision 2030.

Akbar, M., Jena, K. K. and Seshu, D. V. (1987). Salt tolerance in wild rices. *Int. Rice Res. Instit. News Lett.* 12:15.

Alaily, F., Sib, J., Madrid, K., Soubeiga, Z. and Roth, C. (1998). Paddy soils derived from basaltic colluvium in the highland of Madagascar. *J. Plant Nutr. Soil Sci*. 163: 633-637.

Alscher, R. G., Erturk, N. and Heath, L. S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot*. 53: 1331-1341.

Amberger, A. and Mathan, K. K. (1977). Influence of iron on the uptake of phosphorus by maize. *Plantand* S*oil*. 46: 413-422.

Ando, T., Yoshida, S. and Nishiyama, I. (1983). Nature of oxidizing power of rice roots. *Plant and Soil*. 72: 57-71.

Aoyama, T., Kobayashi, T., Takahashi, M., Nagasaka, S., Usuda, K., Kakei, Y., Ishimaru, Y., Nakanishi, H., Mori, S. and Nishizawa, N. K. (2009). OsYSL18 is a rice iron (III)–deoxymugineic acid transporter specifically expressed in reproductive organs and phloem of lamina joints. *Plant Mol. Biol.* 70: 681-692.

Asada, K. (1999). The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Plant Mol. Biol.* 50: 601–639.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol*. 141: 391–396.

Asch, F., Becker, M. and Kpongor, D. S. (2005). A quick and efficient screen for resistance to iron toxicity in lowland rice. *J Plant Nutr Soil Sci.* 168:764–773.

Asch, F., Dingkuhn, M. and Dörffling, K. (2000). Leaf K/Na ratio predicts salinity induced yield loss in irrigated rice. *Euphytica* 113: 109-118.

Asch, F., Dahal, K. P. and Das, U. S. (2003). Is the transpiration history of rice leaves indicative for the salt load of individual leaves? In University of Göttingen and ATSAF: Technological and Institutional innovations for Sustainable Rural Development. Klartext GmbH, Göttingen, Germany, 124.

Armstrong, W. (1979). Aeration in higher plants. *Advances in Botanical Research* 7, 225–332.

Armstrong, J. and Armstrong, W. (2005). Rice: sulfide-induced barriers to root radial oxygen loss, Fe· and water uptake, and lateral root emergence. *Ann. Bot* .96: 625-638.

Audebert, A. and Fofana, M. (2009). Rice yield gap due to iron toxicity in West Africa. *J. Agron. Crop Sci.* 195:66-76.

Audebert, A. and Sahrawat, K. L. (2000). Mechanisms for iron toxicity tolerance in lowland rice. *J. Plant Nutr.* 23:1877-1885.

Awal, M. A. and Ikeda, T. (2002). Recovery strategy following the imposition of episodic soil moisture deficit in stands of peanut (*Arachis hypogaea* L.). *J. Agron. Crop Sci*. 188: 185-192.

Basten, C. J., Weir, B. S. and Zeng, Z. B. (1994). Zmap-a QTL cartographer In: Smith, C., Gavora, J. S., Benkel, B., Chesnais, J., Fairfull, W., Gibson, J. P., Kennedy, B. W., Burnside, E. B. (eds) Proceedings of the 5th World congress on genetics applied to livestock production: computing strategies and software, 22:65–66. Published by the Organizing Committee, 5th world congress on genetics applied to livestock production, Guelph, Ontario.

Basten, C. J., Weir, B. S. and Zeng, Z. B. (2004). QTL Cartographer, Version 1.17. Department of Statistics, North Carolina State University, Raleigh.

Bahiigwa, G., Rigby, D. and Woodhouse, P. (2005). Right target, wrong mechanism? Agricultural modernization and poverty reduction in Uganda. *World Dev*, 33: 481-496.

Bashir K., Hanada K., Shimizu M., Seki M., Nakanishi H. and Nishizawa N. K. (2014). Transcriptomic analysis of rice in response to iron deficiency and excess. *Rice* 7: 18.

Bashir, K. and Nishizawa, N. K. (2006). Deoxymugineic acid synthase;a gene important for Fe-acquisition and homeostasis. *Plant Signal and Behav* 1: 290-292.

Bashir, K., Inoue, H., Nagasak, S., Takahashi, M., Nakanishi, H., Mori, S. and Nishizawa, N. K. (2006). Cloning and characterization of deoximugineic acid synthase genes in graminaceous plants. *J. Biol. Chem* 281: 32395-32402.

Bashir, K., Ishimaru, Y. and Nishizawa, N. K. (2010). Iron uptake and loading into rice grains. *Rice* 3:122-130.

Bashir, K., Ishimaru, Y. and Nishizawa, N. K. (2011a). Identification and characterization of the major mitochondrial Fe transporter in rice. *Plant Signal and Behav* 6:1591-1593.

Bashir, K., Ishimaru, Y., Shimo, H., Kakei, Y., Senoura, T., Takahashi, R., Sato, Y., Uozumi, N., Nakanishi, H.and Nishizawa, N. K. (2011b). Rice phenolics efflux transporter 2 (PEZ2) plays an important role in solubilizing apoplasmic iron. *Soil Sci Plant Nutr* 57:803-812.

Bashir, K., Nozoye, T., Ishimaru, Y., Nakanishi, H. and Nishizawa, N.K. (2013b). Exploiting new tools for iron bio-fortification of rice. *Biotech Advan* 31:1624-1633.

Bashir, K., Takahashi, R., Akhtar, S., Ishimaru, Y., Nakanishi, H. and Nishizawa, N.K. (2013a). The knockdown of OsVIT2 and MIT affects iron localization in rice seed. *Rice* 6:1-7.

Bashir, K., Hanada, K., Shimizu, M., Seki, M., Nakanishi, H. and Nishizawa, N. K. (2014). Transcriptomic analysis of rice in response to iron deficiency and excess. *Rice* 7:18.

Becana, M., Moran, J. F. and Iturbeormaetxe, I. (1998). Iron-dependent oxygen free radical generation in plants subjected to environmental stress: toxicity and antioxidant protection. *Plant and Soil* 201: 137-147.

Becker, M. and Asch, F. (2005). Iron toxicity in rice – conditions and management concepts. J. *Plant Nutr. Soil Sci*. 168:558-573.

Benckiser, G., Ottow, J. C. G., Santiago, S. and Watanabe, I. (1982). Physiochemical characterization of iron-toxic soils in some Asian countries. IRRI Research Paper Series No. 85, *Int Rice Res Inst*, Manila, The Philippines, 11.

Bienfait, H. F. (1985). Regulated redox processes at the plasma-lemma of plant root cells and their function in iron uptake. *J. Pioneer Biomembr*. 17: 73-83.

Bode, K., Oring, O. D.,¨Uthje, S. L., Neue, H. U. and ¨Ottger, M. B. (1995). The role of active oxygen in iron tolerance of rice (*Oryza sativa* L.). *Protoplasma* 184: 249-255.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem*, 72:248–254.

Brady, N. C. (1982). Rice research strategies for the future. The International Rice Research Institute, Manila, The Philippines, 13-25.

Brand-Williams, W., Cuvelier, M. E. and Berste, C. (1995). Use of free radical method to evaluate antioxidant activity. *Technol.* 28: 25-30.

Basten, C. J., Weir, B. S. and Zeng, Z. B. (1994). Zmap-a QTL cartographer In: Smith, C., Gavora, J. S., Benkel, B., Chesnais, J., Fairfull, W., Gibson, J. P., Kennedy, B. W., Burnside, E. B. (eds) Proceedings of the 5th World congress on genetics applied to livestock production: computing strategies and software, 22:65-66. Published by the Organizing Committee, 5th world congress on genetics applied to livestock production, Guelph, Ontario.

Basten, C. J., Weir, B. S. and Zeng, Z. B. (2004). QTL Cartographer, Version 1.17. Department of Statistics, North Carolina State University, Raleigh.

Briat, J. F., Ravet, K., Arnaud, N., Duc, C., Boucherez, J.,Touraine, B., Cellier, F. and Gaymard, F. (2010). New insights into ferritin synthesis and function highlight a link between iron homeostasis and oxidative stress in plants. *Ann Bot*. 7:811-822.

Briat, J. F. and Lebrun, M. (1999). Plant responses to metal toxicity. *Academic Sci. III.* 322: 43-54.

Brundett, M. C., Kendrick, B. and Peterson, C. A. (1991). 'Efficient lipid staining in plant material with Sudan Red 7B or floral yellow 088 in polyethylene glycol-glycerol. *Biotechnol. Histochem*. 66: 111-116.

Brunings, A. M., Datnoff, L. E., Mitani N., Nagamura Y., Rathinasabapathi, B. and Kirst. M. (2009). Differential gene expression of rice in response to silicon and rice blast fungus *Magnaporthe oryzae. Ann, Appl. Biol.*155:161-170.

Chakrabarty, D., Trivedi, P. K., Misra, P., Tiwari, M., Shri, M., Shukla, D., Kumar, S., Rai, A., Pandey, A., Nigam, D., Tripathi, R. D. and Tuli, R. (2009). Comparative transcriptome analysis of arsenate and arsenite stresses in rice seedlings. *Chemosphere*. 74: 688-702.

Cakmak, I. (1988). Morphologische und physiologische Veräaderungen bei Zink-Mangelpflanzen. Ph.D. Thesis, University of Hohenheim, Stuttgart, Germany.

Common Fund for Commodities by the European Cooperative for Rural Development: Rice Sector Development in East Africa (2012).

Chen, C. C., Dixon, J. B. and Turner, F. B. (1980a). Iron coatings on rice leaves: Morphology and models of development. *Soil Sci. Soc. Am. J.* 44:1113-1119.

Chen, C. C., Dixon, J. B. and Turner, F. B. (1980b). Iron coatings on rice leaves: Mineralogy and quantity influencing factors. *Soil Sci. Soc. Am. J.* 44:635-639.

Chao, D. Y., Luo, D. and Lin H. X. (2005). Salt responsive genes in micro array analysis. *Cell Res*. 15. 796-810.

Colmer, T. D. (2003a). Aerenchyma and an inducible barrier to radial oxygen loss facilitate root aeration in upland, paddy and deep-water rice (*Oryza sativa* L.). *Ann. Bot*.91: 301-309.

Colmer, T. D. (2003b). Long-distance transport of gases in plants: a perspective on internal aeration and radial oxygen loss from roots. *Plant, Cell Environ*. 26: 17-36.

Conte, S. S. and Walker, E. L. (2011). Transporters contributing to iron trafficking in plants. *Mol Plant*. 4: 464-476.

Couée, I., Sulmon, C., Gouesbet, G. and Amrani, A. E. (2006). Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *J. Exp. Bot*. 57: 449-459.

Curie, C. and Briat, J. F, (2003). Iron transport and signaling in plants. *Annual Review of Plant Biol.* 54:183-206.

Curie, C., Cassin, G., Couch, D., Divol F., Higuchi, K., Jean, M. L., Misson, J., Adam S., and Stéphane, M. (2009). Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Ann of Bot*, 103:1-11.

Degenkolbe, T., Do, P.T., Zuther, E., Repsilber, D., Walther, D., Hincha, D.K. and Kohl, K.I. (2009). Expression profiling of rice cultivars differing in their tolerance to long-term drought stress. *Plant Mol. Biol*, 69, 133-153.

Deng, H., Ye, Z. H. and Wong, M. H. (2009). Lead, zinc and iron ($Fe2^{+}$) tolerances in wetland plants and relation to root anatomy and spatial pattern of ROL. *Environ. Exp Bot*. 65: 353-362.

Deturck, P. (1994). I ron toxicity to rainfed lowland rice in Sri Lanka. *KULFLTB*, Leuven, Belgium.

De Simone, O., Haase, K., Müller, E., Junk, W. J., Hartmann, K., Schreiber, L. and Schmidt, W. (2003). Apoplasmic barriers and oxygen transport properties of hypodermal cell walls in roots from four Amazonian tree species. *Plant Physiol*. 132: 206-217.

Dingkuhn, M., Audebert, A., Jones, M. P., Etienne, K. and Sow, A. (1999). Control of stomatal conductance and leaf rolling in *O. sativa* and *O. glaberrima* upland rice. *Field Crops Res*. 61: 223-236.

Dobermann, A. and Fairhurst, T. H. (2000). Nutrient disorders and nutrient management. The *Int Rice Res Inst*, Manila, The Philippines, 191.

Dufey, I., Hakizimana, P., Draye, X., Lutts, S. and Bertin, P. (2009). QTL mapping for biomass and physiological parameters linked to resistance mechanisms to ferrous iron toxicity in rice. *Euphytica.* 167:143-160.

Dufey, I., Hiel, M.P., Hakizimana, P., Draye, X., Lutts, S., Kone,´ B., Drame,´ K.N., Konate´, K.A., Sie,´ M and Bertin, P. (2012a). Multienvironment QTL mapping and consistency across environments of resistance mechanisms to ferrous iron toxicity in rice. *Crop Sci.* 52 :539-550.

Dufey, I., Draye, X., Lutts, S., Lorieux, M., Martinez, C. and Bertin, P. (2012b). QTL mapping for resistance to ferrous iron toxicity in rice using an interspecific backcross Oryza sativa x Oryza glaberrima. In: Dufey I Genetic determinism of resistance mechanisms to ferrous iron toxicity in rice. Ph.D. thesis, Universite´ catholique de Louvain (UCL), Louvain-La-Neuve, Belgium, 173.

Dufey I., Mathieu A.S., Draye X., Lutts S., Bertin P. (2015a). Construction of an integrated map through comparative studies allows the identification of candidate regions for resistance to ferrous iron toxicity in rice. *Euphytica*. 203: 59-69.

Dufey I., Draye X., Lutts S., Lorieux M., Martinez C., BertinP. (2015b). Novel QTLs in an interspecific backcross *Oryza sativa* × *Oryza glaberrima* for resistance to iron toxicity in rice. *Euphytica.*

Engel, K. (2009). Efficiency of adaptation mechanisms of rice to diverse conditions of iron toxicity. eScholarship University of California.

Engel, K., Asch, F. and Becker, M. (2012). Classification of rice genotypes based on their tolerance and the mechanisms of adaptation to conditions of iron toxicity. *J. Plant Nutr. Soil Sci*. 7:871-881.

Fairhurst, T. H. and Witt, C. (2002). Rice: A practical guide to nutrient management.
International Rice Research Institute (IRRI), Manila

Fales, F. W. (1951). The assimilation and degradation of carbohydrates by yeast cells. *J. Biol. Chem.* 193:113-124.

Famoso, A. N., Randy, T. C., Jon, E. S., Eric, C., Susan, R. M. and Kochian, L. V. (2010). Development of a novel aluminum tolerance phenotyping platform used for comparisons of cereal aluminum tolerance and investigations into rice aluminum tolerance mechanisms. *Plant Physiol*. 153:1678-1691.

Fang,W. C. and Kao, C. H. (2000). Enhanced peroxidase activity in rice leaves in response to excess iron, copper and zinc. *Plant Sci.*158:71-76.

Fang, W. C., Wang, J.W., Lin, C. C. and Kao, C. H. (2001). Iron induction of lipid peroxidation and effects on antioxidative enzyme activities in rice leaves. *Plant Growth Regul.* 35:75-80.

FAO (2010). The State of Food Insecurity in the World Addressing food insecurity in protracted crises. Rome, FAO.

FAO (2015) Global Initiative on Food Loss and Waste Reduction. Rome, FAO.

FAO (2018). The State of Food Security and Nutrition in the World 2018. Building climate resilience for food security and nutrition. Rome, FAO.

Finatto, T., de Oliveira, A. C., Chaparro, C., da Maia, L. C., Farias, D. R., Woyann, L. G., Mistura, C. C., Soares-Bresolin, A. P., Llauro, C., Panaud, O. and Picault, N. (2015). Abiotic stress and genome dynamics: specific genes and transposable elements response to iron excess in rice. *Rice*8:13.

Franke, R. and Schreiber, L. (2007). Suberin a biopolyester forming apoplastic plant interfaces. Curr. *Opinion Plant Biol*. 10: 252-259.

Frei, M., Tetteh, R. N., Razafindrazaka, A. L. (2016). Responses of rice to chronic and acute iron toxicity: genotypic differences and biofortification aspects. *Plant Soil*. 408: 149-161.

Fukuda, A., Shiratsuchi, H., Fukushima, A., Yamaguchi, H., Mochida, H., Terao, T., Ogiwara, H. (2012). Detection of chromosomal regions affecting iron concentration in rice shoots subjected to excess ferrous iron using chromosomal segment substitution lines between *Japonica* and *Indica*. *Plant Prod. Sci.* 15:183-191.

Gallie, D. R. (2013). The role of l-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. *J. Exp. Bot*. 7:433-443.

Garrity, D. P., Oldeman, L. R., Morris, R. A. and Lanka, D. (1986). Rainfed lowland rice ecosystems, in IRRI: characterization and distribution: progress in rainfed lowland rice. International Rice Research Institute: Manila, The Philippines.

Garthwaite, A. J., Armstrong, W. and Colmer, T. D. (2008). Assessment of O_2 diffusivity across the barrier to radial O_2 loss in adventitious roots of *Hordeum marinum*. *New Phytol*. 179: 405-416.

Genon, J.G., Hepcee, N., Duffy, J.E., Delvaux, B. and Hennebert, P.A. (1994). Iron toxicity and other chemical constraints to rice land swamps of Burundi. *Plant and Soil* 166: 109-115.

Gechev, T. S., Van Breusegem, F., Stone, J. M., Denev, I. and Laloi, C. (2006). Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bio Essays*. 28: 1091-1101.

Geiger, D. R., Servaites, J. C. and Fuchs, M. A. (2000). Role of starch in carbon translocation and partitioning at the plant level. *Aust. J Plant Physiol*. 27: 571-582.

Gibon, Y. (2004). Adjustment of diurnal starch turnover to short days: Depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post-translational activation of ADP-glucose pyrophosphorylase in the following light period. *Plant J*. 39: 847-862.

Goicoechea, N., Aguirreolea, J., Cenoz, S. and García-Mina, J. M. (2001). Gas exchange and flowering in Verticillium -wilted plants. *J. Phyto-pathol*. 149: 281-286.

Green, M. S., and Etherington, J. R. (1977). Oxidation of ferrous iron by rice (Oryza sativa L.) roots: A mechanism for waterlogging tolerance? *J Exp Biol*. 28: 678-690.

Greenway, H., Armstrong, W., and Colmer, T. D. (2006). Conditions leading to high CO2 (>5 kPa) in waterlogged-flooded soils and possible effects on root growth and metabolism. *Ann. Bot*.98: 9-32.

Gregorio, G. B., Senadhira, D., Htut, H., Graham, D. (2000). Breeding for trace mineral density in rice. Food and Nutrition Bulletin, 21: 382-386.

Grotz, N. and Guerinot M. L. (2006) Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochem Biophys Acta*.1763: 595-608.

Guerinot, M. L. and Yi, Y. (1994). Iron: nutritious, noxious, and not readily available. *Plant Physiol.* 104: 815-820.

Gunawardena, I., Virmani, S. and Sumo, F. J. (1982). Breeding rice for tolerance to iron toxicity. *Oryza* 19: 5-12.

Gupta, A. S., Webb, R. P., Holaday, A. S. and Allen, R. D. (1993). Over expression of superoxide dismutase protects plants from oxidative stress. *Plant Physiol*. 103: 1067-1073.

Gupta, A. K. and Kaur, N. (2005). Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *J. Biosci*. 30: 761-76.

Haefele, S, M., Nelson, A., Hijmans, R. J. (2014). Soil quality and constraints in global rice production. *Geoderma*. 235:250-259.

Hell, R. and Stephan, U.W. (2003). Iron uptake, trafficking and homeostasis in plants. *Planta.* 216: 541-551.

Hodges, D. M., DeLong, J. M., Forney, C. F. and Prange, R. K. (1999). Improving the thiobarbituric acid-reactive substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 207: 604-611.

Huke, R.E. and Huke, E.H. (1997). Rice area by type of culture-in South, Southeast, and East Asia. A revised and updated data base. IRRI, Los Baños, Philippines.

Inouée, H., Higushi, K., Takahashi, M., Nakanishi, H., Mori, S. and Nishizawa, N. K. (2003). Three rice nicotianamine synthase genes, *OsNAS1*, *sNAS2*, and *OsNAS3* are expressed in cells involved in long-distance transport of iron and differentially regulated by iron. *The Plant J.* 36: 366-381.

Inouée, H., Takahashi, M., Kobayashi, T., Suzuki, M., Nakanishi, H., Mori, S. and Nishizawa, N. K. (2008). Identification and localization of the rice nicotianamine aminotransferase gene *OsNAAT1*expression suggests the site of phytosiderophore synthesis in rice. *Plant Mol Biol.* 66: 193-203.

Inouée, H., Kobayashi, T., Nozoye, T., Takahashi, M., Kakei, Y., Suzuki, K., Nakazono, M., Nakanishi, H., Mori, S. and 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. *J Biol Chem.* 284: 3470-3479.

Insalud, N., Bell, R. W., Colmer, T. D. and Rerkasem, B. (2006). Morphological and physiological responses of rice (*Oryza sativa*) to limited phosphorus supply in aerated and stagnant solution culture. *Ann. Bot*. 98: 995-1004.

IRRI. (2002). Standard evaluation system (SES) for rice. Manila, Philippines.

IRRI (2012) Annual Report. Rice science for a better world.

IRRI (2013): Introducing "Statistical Tool for Agricultural Research (STAR)"

Ishimaru, Y., Suzuki, M., Tsukamoto, T., Suzuki, K., Nakazono, M., Kobayashi, T., Wada, Y., Watanabe, S., Matsuhashi, S., Takahashi, M., Nakanishi, H., Mori, S. and Nishizawa, N. K. (2006). Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. *Plant J.* 45: 335-346.

Ishimaru, Y., Bashir, K., Fujimoto, M., An, G., Itai, R.N., Tsutsumi, N., Nakanishi, H. and Nishizawa, N.K. (2009). Rice-specific mitochondrial iron–regulated gene (MIR) plays an important role in iron homeostasis. *Mol Plant* 2:1059-1066.

Ishimaru, Y., Takahashi, R., Bashir, K., Shimo, H., Senoura, T., Sugimoto, K., Ono, K., Yano, M., Ishikawa, S., Arao, T., Nakanishi, H. and Nishizawa, N. K. (2012). Characterizing the role of rice NRAMP5 in manganese, iron and cadmium transport. *Sci Rep* 2:286.

Ismail, A., M. (2018). Submergence tolerance in rice: resolving a pervasive quandary. *New Phytologist* 218:1298-1300.

Jackson, M. B. and Armstrong, W. (1999). Formation of aerenchyma and the processes of plant ventilation in relation to soil flooding and submergence. *Plant Biol*. 1: 274-287.

Jensen, W. A. (1962). Botanical histochemistry-principles and practice, W.H Freeman, San Francisco, Calif, USA.

Johansen, D. A. (1940). Plant microtechnique. New York, NY, USA: McGraw-Hill Book Co., Inc.

Kampfenkel. K., Van, M. V. and Inze,´ D. (1995). Extraction and determination of ascorbate and dehydroascorbate from plant tissue. *Anal. Biochem*. 225: 165-167

Kang, D. J., Koichi, F., Young-Jin, S., Pisoot, V. and Ryuichi, I. (2011). Relationship of Fe-tolerance to morphological changes in roots in upland NERICA lines under Fe-treated hydroponic conditions. *Crop Sci*. 4: 311-315.

Kao, C. H., Fang, W. C., Wang, J. W., Lin, C. C. (2001). Iron induction of lipid peroxidation and effects on antioxidative enzyme activities in rice leaves. *Plant Growth Regul*. 1: 75-80.

KARI (2009). The Kenya Agricultural Research Institute 2010-2014 strategic plan and its implementation framework.

Kawase, M. (1981). Automatical and morphological adaptation of plants to water logging. *Hortsci*. 16:30-34.

Kim, S. A., Guerinot, M. (2007). Mining iron: iron uptake and transport in plants. LFEBS Letters 581:2273-80.

.

Kim SH, Song M, Lee K, Hwang SG, Jang C, Kim JB, Kim S, Ha BK, Kang SY, Kim D. (2012). Genome-wide transcriptome profiling of ROS scavenging and signal transduction pathways in rice (Oryza sativa L.) in response to different types of ionizing radiation. *Mol Biol Rep*.39:11231-11248

Kobayashi T, Itai RN, Nishizawa NK (2014) Iron deficiency responses in rice roots. *Rice*7:27.

Kobayashi, T. and Nishizawa, N.K. (2008). Regulation of iron and zinc uptake and translocation in rice. In *Rice Biology in the Genomics Era. Biotechnology in Agriculture and Forestry, 62* (eds H.Y. Hirano, Y. Sano, A. Hirai and T. Sasaki), 321-335. Springer- Verlag, Berlin Heidelberg.

Koike S, Inoue H, Mizuno D, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2004) *OsYSL2* is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. *The Plant Journal.* 39:415-424

Kosegarten, H., Hofmann, B. and Mengel, K. (1999). Apoplastic pH and Fe3+-reduction in intact sunflower leaves. *Plant Physiol*. 121:1069-1079.

Kotula, L., Ranathunge, K., Schreiber, L. and Steudle, E. (2009). Functional and chemical comparison of apoplastic barriers to radial oxygen loss in roots of rice (*Oryza sativa*L.) grown in aerated or deoxygenated solution. *J. Exp. Bot.* 60: 2155-2167.

Krämer, U., Talke, I. N., Hanikenne, M. (2007) Transition metal transport. *FEBS Lett*. 581: 2263-2272.

Krishnamurthy, P., Ranathunge, K., Franke, R., Prakash, H. S., Schreiber, L. and Mathew, M. K. (2009). The role of root apoplastic transport barriers in salt tolerance of rice (*Oryza sativa*L.). *Planta.*230:119-134.

Kuki, K. N., Oliva, M. A., Pereira, E. G., Costa, A. C. and Cambraia, J. (2008). Effects of simulated deposition of acid mist and iron ore particulate matter on photosynthesis and the generation of oxidative stress in *Schinus terebinthifolius* radii and *Sophora tomentosa* L. *Sci. Total Environ.* 403: 207-214.

Kumar, P., Tewari, R. K. and Sharma, P. N. (2008). Modulation of copper toxicity-induced oxidative damage by excess supply of iron in maize plants. *Plant Cell Rep*. 27:399-409.

Landsberg, E. C. (1996). Hormonal regulation of iron-stress response in sunflower roots: a morphological and cytological investigation. *Protoplasma.* 194:69-80.

Larson, R.A. (1988). The antioxidants of higher plants. *Phytochem* 27:969-978.

Lee, S., Chiecko, J.C., Kim, S.A., Walker, E.L., Lee, Y., Guerinot, M.L. and An, G. (2009). Disruption of OsYSL15 Leads to iron inefficiency in rice plants. *Plant Physiol* 150:786–800.

Li, S., Yang, W., Yang, T., Chen, Y. and Nia, W. (2015). Effects of cadmium stress on leaf chlorophyll fluorescence and photosynthesis of *Elsholtzia argyi* - a cadmium accumulating plant. *Int. J. Phytore-mediat.* 17:85-92.

Lichtenthaler, H. K. and Buschmann, Claus. (2001). Chlorophylls and carotenoids: measurement and characterization by UV-VIS pectroscopy. Current Protocols in Food Analytical Chemistry. F4.3.1-F4.3.8.

Lowry, o. H. and Lopez, J. A. (1945). The colorimetric determination of phosphorus. *J. Biol. Chem*. 66:375-400.

MacLean, S. J., Andrews, B.C. and Verheyen, E.M. (2002).Characterization of Dir: a putative potassium inward rectifying channel in Drosophila .*Mech. Dev*. 116: 193-197.

Magnavaca, R., Gardner, C. O. and Clark, R. B. (1987). Evaluation of inbred maize lines for aluminum tolerance in nutrient solution. In Genetic Aspects of Plant Mineral Nutrition. Martinus Nijhoff, Dordrecht. *The Netherlands*. 255-265.

Mahadevappa, M., Bulbule, A. V., Siddaramappa, R. and Kulkarni, R. S. (1991). Field screening of rice for growth, yield and nutrient uptake in acidic soil. Develop. *Plant Soil Sci.* 45:959-965.

Majerus, V., Bertin, P. and Lutts, S. (2007). Effects of iron toxicity on osmotic potential, osmolytes and polyamine concentration in the African rice (*Oryza glaberrima*Steud). *Plant Sci*. 173: 96-105.

Marschner, H. (1995). Mineral nutrition of higher plants. London, UK: Academic Press.

Matthus, E., Wu, L. B., Ueda, Y., Höller, S., Becker, M., Free, M. (2015). Loci, genes, and mechanisms associated with tolerance to ferrous iron toxicity in rice (*Oryza sativa* L.). *Theoret and Appl. Gene.*128: 2085-2098.

McDonald, D. J., (1979). Rice Chapter 3. In: Australian field crops: Oilseeds, grain legumes and other crops. Angus and Robertson. London.

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trendsin Plant Sci.*7:405-410.

Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends Plant Sci*. 9:490-498.

Ministry of Agriculture (2008). Rice in Kenya

Ministry of Agriculture (2010). Rice in Kenya

Moradi, F. and Ismail, A., M. (2007). Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. Ann. Bot. 99: 1161-1173.

Morrissey J, Guerinot ML (2009) Iron uptake and transport in plants: the good, the bad, and the ionome. *Chemical Reviews* 109, 4553–4567.

Müller, C., Kuki, K.N., Pinheiro, D.T., de Souza, L. R., Silva, A. I. S., Loureiro, M. E., Oliva, M. A and Almeida, A. M. (2015). Differential physiological responses in rice upon exposure to excess distinct iron forms. *Plant Soil.* 391: 123-138.

Neves, N. R., Oliva, M. A., Centeno, D. C., Costa, A. C., Ribas, R. F. and Pereira, E. G. (2009). Photosynthesis and oxidative stress in the resting plant species Eugenia uniflora L. exposed to

simulated acid rain and iron ore dust deposition: potential use in environmental risk assessment. *Sci. Total Environ.* 407:3740-3745.

Netondo, G. W., J. C. Onyango and Beck, E. (2004). Response of growth, water relations, and ion accumulation to NaCl salinity. *Crop Sci*. 44: 797-805.
NIBS (2008). National Irrigation Board strategies plan 2008-2013. Government of Kenya.

Nicolic, M. and Römheld, V. (2001). The role of the leaf apoplast in iron nutrition of plants, in Horst, W. J. et al.: Plant Nutrition – food security and sustainability of Agro-Ecosystems. Kluwer Academic Publishers, Dordrecht. The Netherlands. 274-275.

Noreen, Z. and Ashraf, M. (2009). Assessment of variation in antioxidative defense system in salt-treated pea (Pisum sativum) cultivars and its putative use as salinity tolerance markers. *J. Plant Physiol.*166: 1764-1774.

Nozoe T., Agbisit, R., Fukuta, Y., RODRIGUEZ, R. and Yanagihara, S. (2008). Characteristics of Iron Tolerant Rice Lines Developed at IRRI under Field Conditions. *JARQ*. 42: 187-192.

Nozoye, T., Nagasaka, S., Kobayashi, T., Takahashi, M., Sato, Y., Sato, Y., Uozumi, N., Nakanishi, H. and Nishizawa, N.K. (2011). Phytosiderophore efflux transporters are crucial for iron acquisition in graminaceous plants. *J Biol Chem* 286: 5446-5454.

Nozoye, T., Nagasaka, S., Bashir, K., Takahashi, M., Kobayashi, T., Nakanishi, H., Nishizawa, N. K. (2014). Nicotianamine synthase 2 localizes to the vesicles of iron-deficient rice roots, and its mutation in the YXXj or LL motif causes the disruption of vesicle formation or movement in rice. *The Plant J. l*77: 246 -260.

NRDS, (2009). Kenya National Rice Development strategic plan 2009 to 2013 "The roadmap to self- sufficiency in rice production.

Nwanze, K. F., Savitri M., Patrick, K., Shellemiah, K. and Samuel, B. O. (2006). Rice Development in Sub-Saharan Africa. *J. Sci. Food and Agric*. 86: 675-677.

Obata, H., Inouhe N. and Umebayashi, M. (1996). Effect of Cd on plasma membrane ATPase from plant roots differing in tolerance to Cd. *Soil Science and Plant Nutr.*42: 361-366.

OECD, (1999). Consensus document on the biology of Oryza sativa (Rice).ENV/JM/MOMO (99) 26. Organization for Economic and coo-operation and Development.

Onaga, G., Edema, R. and Asea, G. (2012). Tolerance of rice germplasm to iron toxicity stress and the relationship between tolerance, $Fe2^{+}$, P and K content in the leaves and roots. *Arch. Agron. Soil Sci*. 59:213-219.

Onaga, G., Edema, R. and Asea, G. (2013*a*). Tolerance of rice germplasm to iron toxicity stress and the relationship between tolerance, $Fe2^{+}$, P and K content in the leaves and roots. *Arch. Agron. Soil Sci.*59: 213-229.

Onaga, G., Dramé, K. N. and Ismail, A. (2016). Understanding the regulation of iron nutrition: can it contribute to improving iron toxicity tolerance in rice? *Funct Plant Biol.* 43: 709-726.

Ottow, J. C. G., Benckiser, G. and Watanabe, I. (1982). Iron toxicity of rice as a multiple nutritional stress. *Tropical Agric Res Series.* 15: 167-179.

Pantin, F., Simonneau, T., Rolland, G., Dauzat, M. and Muller, B. (2011). Control of leaf expansion: a developmental switch from metabolics to hydraulics. *Plant Physiol*. 156: 803-815.

Peng, X. X. and Yamauchi, M. (1993). Ethylene production in rice bronzing leaves induces by ferrous iron. *Plant and Soil.* 149: 227-234.

Pereira, E. G., Oliva, M. A., Rosado-Souza, L., Mendes, G. C., Colares, D. S., Stopato., C. H. and Almeida, A. M. (2013). Iron excess affects rice photosynthesis through stomatal and non-stomatal limitations. *Plant Sci.* 201: 81-92.

Pereira, E. G., Oliva, M. A., Siqueira-Silva, A. I., Rosado-Souza, L., Pinheiro, D. T. and Almeida, A. M. (2014). Tropical rice cultivars from lowland and upland cropping systems differ in iron plaque formation. *J. Plant Nutr*. 37: 1373-1394.

Ponnamperuma, F. N. (1977). Physico-chemical properties of submerged soils in relation to fertility. *IRRI Research Paper Series* 5:32.

Ponnamperuma, F. N. and Solivas, J. (1982). Field amelioration of an acid sulfate soil for rice with manganese dioxide and lime. In Proceedings of the Bangkok symposium on acid sulfate soils. H. Dost and N. van Breemen, eds. ILRI Publ. 31. ILRI, Wageningen, The Netherlands.

Ptashnyk, M, Roose, T, Jones, D. L and Kirk, G. J. D. (2011). Enhanced zinc uptake by rice through phytosiderophore secretion: a modelling study. *Plant, Cell & Environ.* 34: 2038-2046.

Qian, H., Wang, R., Chen, J., Ding, H., Yong, W., Songlin, R. and Fu, Z. (2012). Analysis of enantioselective biochemical, physiological, and transcriptional effects of the chiral herbicide diclofop methyl on rice seedlings. *J. Agric Food Chem* 2012.

Quinet, M., Vromman, D., Clippe, A., Bertin, P., Lequeux, H., Dufey, I., Lutts, S. and Lefe`vre, I. (2012). Combined transcriptomic and physiological approaches reveal strong differences between short and long term response of rice (Oryza sativa) to iron toxicity. *Plant Cell Environ.* 35: 1837-1859.

R Foundation (2015). R Consortium for statistical computing.

Ranathunge, K., Lin, J., Steudle, E. and Schreiber, L. (2011). Stagnant deoxygenated growth enhances root suberization and lignifications, but differentially affects water and NaCl permeabilities in rice (Oryza sativa L.) roots. *Plant Cell Environ*. 34: 1223-1240.

Rao, A.S.V.C., Reddy, A.R. (2008). Glutathione Reductase: A Putative Redox Regulatory System in Plant Cells. In: Khan N.A., Singh S., Umar S. (eds) Sulfur Assimilation and Abiotic Stress in Plants. Springer, Berlin, Heidelberg.

Roitsch, T. (1999). Source-sink regulation by sugar and stress. *Curr. Opin. Plant Biol*. 2: 198-206.

Romero, L. E., Lozano, I., Garavito, A., Carabali, S. J., Triana, M., Villareal, N., Reyes, L., Duque, M. C., Martinez, C. P., Calvert, L. and Lorieux, M. (2014). Major QTLs control resistance to rice hojablanca virus and its vector *Tagosodesorizicolus*. *Genes, Genomes, Genetics* 4: 133-142.

Rout, G. R., Sunita, S., Das, A. B. and Das, S. R. (2014). Screening of iron toxicity in rice genotypes on the basis of morphological, physiological and biochemical analysis. *J. Exp. Biol. Agric. Sci.* 2: 567-582.

Saikia, T. and Baruah, K. K. (2012). Iron toxicity tolerance in rice (*Oryza sativa*) and its association with anti-oxidative enzyme activity. *J. Crop Sci*. 3: 90-94.

.Sahrawat, K. L. and Diatta, S. (1995). Nutrient management and season affect soil iron toxicity. *Annual Report, 1994*. In *West Africa Rice Development Association*, Bouaké, Côte de Ivoire. 34-35.

Sahrawat, K. L. and Singh, B. N. (1997). Management of Iron Toxic Soils for Rice Cultivation in West Africa, In Proceedings of Third African Soil Science Society Conference on Rehabilitation and Management of African Soils for Sustainable Productivity and Environmental Protection, University of Ibadan: Ibadan, Nigeria. 617-624.

Sahrawat, K. L. (2000). Elemental composition of rice plants as affected by iron toxicity under field conditions. Communications in *Soil Sci. and Plant Anal.,* 31: 2819-2827.

Sahrawat, K. L. (2003). Iron toxicity in wetland rice: occurrence and management through integration of genetic tolerance with plant nutrition. *J. Indian Society Soil Sci.* 51: 409-417.

Sahrawat, K. L. (2005) Iron toxicity in wetland rice and the role of other nutrients. *Journal of Plant Nutr.*27, 1471–1504.

Sahrawat, K. L. (2008). Factors Affecting Nitrification in Soils. Communications in *Soil Sci. and Plant Anal.* 39: 1436–1446.

Sairam, R. K. and Saxena, D. C. (2000). Oxidative stress and antioxidants in wheat genotypes: possible mechanism of water stress tolerance. *J. Agron Crop Sci*. 184: 55-61.

Sandalio, L. M., Dalurzo, H. C., Gómez, M., Romero-Puertas, M. C., del Río, L. A. (2001). Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J. Exp Bot.* 52:2115-2126.

Schmidt, W. (1999). Mechanisms and regulation of reduction-based iron uptake in plants. *New Phytol.* 141: 1-26.

Seckback, J. (1982). Ferreting out the secrets of plant ferritin - A review, *J. Plant Nutri*,369: 4-7.

Shimizu, A. (2009). QTL analysis of genetic tolerance to iron toxicity in rice (Oryza sativa L.) by quantification of bronzing score. *J. New Seeds* 10: 171-179.

Shimizu, A., Guerta, C.Q., Gregorio, G.B. and Ikehashi, H. (2005). Improved mass screening of tolerance to iron toxicity in rice by lowering temperature of culture solution. *J Plant Nutr.* 28: 1481-1493.

Shiono, K., Ogawa, S., Yamazaki, S., Isoda, H., Fujimura, T., Nakazono, M. and Colmer, T. D. (2011). Contrasting dynamics of radial O2-loss barrier induction and aerenchyma formation in rice roots of two lengths. *Ann. of Bot.* 107: 89-99.

Shiono, K., Yamauchi, T., Yamazaki, S., Mohanty, B., Malik, A. I., Nagamura, Y., Nishizawa, N. K., Tsutsumi, N., Colmer,T. D. and Nakazono, M. (2014). Microarray analysis of laser-

microdissected tissues indicates the biosynthesis of suberin in the outer part of roots during formation of a barrier to radial oxygen loss in rice (*Oryza sativa*).*J. Exp. Bot.*65: 4795-4806.

Silveira, V. C., Oliveira, A. P., Sperotto, R. A., Amaral, L., Dias, J. F., Cunha, J. B. and Fett, J. P. (2007). Influence of iron on mineral status of two rice (*Oryza sativa* L.) cultivars. Braz. *J. Plant Physiol*. 19: 127-139.

Sikirou, M., Saito, K., Achigan-Dako, E. G., Dramé, K. N., Ahanchédé, A. and Venuprasad, R. (2015). Genetic improvement of iron toxicity tolerance in rice – progress, challenges and prospects in West Africa. *Plant Prod. Sci*.18: 423-434.

Sinha, S. and Saxena, R. (2006). Effect of iron on lipid peroxidation, and enzymatic and non-enzymatic antioxidants and bacoside-A content in medicinal plant *Bacopa monnieri* L. *Chemosphere* 62: 1340-1350.

Smith, A. M. and Stitt, M. (2007). Coordination of carbon supply and plant growth. *Plant Cell Environ.*30: 1126-1149.

Smith, B. N. (1984). Iron in higher plants: Storage and metabolic role. *J. Plant Nutr*. 7:759-766.

Souza-Santos, P., Ramos, R. S., Ferreira, S. T. and Carvalho-Alves, P. C. (2001). Iron-induced oxidative damage of corn root plasma membrane H?-ATPase. *Biochem. Biophys. Acta* 1512: 357-360.

Stein, R. J., Duarte, G. L., Spohr, M.G., Lopes, S.I.G. and Fett, J. P. (2009). Distinct physiological responses of two rice cultivars subjected to iron toxicity in the field. *Ann. Appl. Biol*. 154: 269-277.

Stein, R. J., Irac¸U, S. I. U., Lopes, G. and Fett, J. P. (2014). Iron toxicity in field-cultivated rice: contrasting tolerance mechanisms in distinct cultivars. *Theor. Exp. Plant Physiol*. 26: 135-146.

Streb, S. and Samuel, C. Z. (2012). Starch metabolism in *Arabidopsis*. *Plant Biol*. 26: 135-146.

Suh, H. J., Kim, C.S., Lee, J.Y. and Jung, J. (2002). Photodynamic effect of iron excess on photosystem II function in pea plants. *Photochem. Photobiol*. 75: 513-518.

Sulpice, R., Pyl, E.T., Ishihara, H., Trenkamp, S., Steinfath, M., Witucka- Wall, H., Gibon, Y., Usadel, B., Poree, F., Piques, M.C., Von Korff, M., Steinhauser, M.C., Keurentjes, J.J., Günther, M., Höhne, M.,Selbig, J., Fernie, A.R., Altmann, T. and Stitt, M. (2009). Starch as a major integrator in the regulation of plant growth. USA. *Proc. Natl. Acad. Sci*. 106: 10348-10353.

Tadano, T. (1975). Devices of rice roots to tolerate high iron concentration in growth media. Japan Agricultural Research Quarterly 9:34-39.

Takahashi, R., Ishimaru, Y., Senoura, T., Shimo, H., Ishikawa, S., Arao, T., Nakanishi, H., Nishizawa, N. K. (2011). The OsNRAMP1 iron transporter is involved in Cd accumulation in rice. *J. Expt Bot.* 62: 4843-4850.

Tanaka, A., Loe, R.and Navasero. S. A. (1966). Some mechanisms involved in the development of iron toxicity symptoms in the rice plant. *Soil Sci. Plant Nutr.* 12: 32-38.

Tanaka, A. and Tadano, T. (1969). Iron excluding capacity of rice plants. *J. Sci Soil ManureJpn*. 40:469-472.

Thomson, J. E., Legge, R. L. and Barber, R. F. (1987). The role of free radicals in senescence and wounding. *New Phytol*.105: 317-344.

Thongbai, P. and Goodman, B. A. (2000). Free radical generation and postanoxic injury in rice grown in an iron-toxic soil. *J. Plant Nutr.* 23: 1887-1900.

Tomlinson, K. L., McHugh, S., Labbe, H., Grainger, J. L., Jame, L. E., Pomeroy, K. M., Mullin, J. W., Miller, S. S., Dennis, D. T. and Miki, B. L. A. (2004). Evidence that the hexose-to-sucrose ratio does not control the switch to storage product accumulation in oilseeds: analysis of tobacco seed development and effects of overexpressing apoplastic invertase. *J. Exp Bot*. 55: 2291-2303.

USDA(2016) GRAIN Report: EAC Corn, Wheat and Rice Report

Van Breemen, N. and Moormann, F. R. (1978). Rice: Soil, Water, Land; *Int Rice Res Inst*: Manila, The Philippines.

van Breemen, N. and Pons, L. J. (1978). Acid sulfate soils and rice. Pages 739-761 in Soils and rice. International Rice Research Institute, Los Baños, Philippines.

van de Mortel, J. E., Villanueva, L. A., Schat, H., Kwekkeboom, J., Coughlan, S., Moerland, P. D., van Themaat, E. V. L., Koornneef, M. and Aarts, M. G. M. (2006). Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana*and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiol*. 142:1127-1147.

van Oort, P. A. J. (2018). Mapping abiotic stresses for rice in Africa : drought, cold, iron toxicity, salinity and sodicity. *Field Crops Res*.219: 55-75.

Vaughan, D. A. (1994).Wild relatives of rice: Genetic Reseources handbook. Rice Research Institute, Manila, The Philippines. 7:137.

Vaughan, D. A. (2003).Diversity in the *Oryza* genus. Current Opinion in *Plant Biol*. 6:139-146.

Venus, E. H. (2008). Standardization of screening technique and QTL Analysis for iron toxicity tolerance in rice (Oryza sativa). MS Thesis (Plant Breeding), University of the Philippines, Los Baños, College, Laguna. 133pp.

Venus, E., Quimio, C. A., Mendoza, R., Sajise, A. G. C., Beebout, S. E. J., Gregorio, G. B. and Singh, R. K. (2013). Maintaining elevated Fe^{2+} concentration in solution culture for the development of a rapid and repeatable screening technique for iron toxicity tolerance in rice (Oryza sativa L.). *Plant Soil*. 372: 253-264.

Vision (2030). Government of Kenya Vision 2030 report

Visser, E. J. W., Colmer, T. D., Blom, C. W. P. M. and Voesenek, L. A. C. J. (2000). Changes in growth, porosity, and radial oxygen loss from adventitious roots of selected mono- and dicotyledonous wetland species with contrasting types of aerenchyma. *Plant Cell Environ*. 23: 1237-1245.

Vose, P. B. (1983). Rationale of selection for specific nutritional characters in crop improvement with *Phaseolus vulgaris* L. as a case study. *Plant Soil.* 72: 351-364.

Wan, J. L., Zhai, H. Q., Wan, J. M. and Ikehashi, H. (2003a). Detection and analysis of QTLs for ferrous iron toxicity tolerance in rice, Oryza sativa L. *Euphytica.* 131: 201-206.

Wan, J. L., Zhai, H. Q., Wan, J. M., Yasui, H. and Yoshimura, A. (2003b). Mapping QTL for traits associated with resistance to ferrous iron toxicity in rice (Oryza sativa L.), using japonica chromosome segment substitution lines. *Yi Chuan Xue Bao.* 30: 893-898.

Wan, J. L., Zhai, H. Q., Wan, J. M., Yasui, H. and Yoshimura, A. (2004). Detection and analysis of QTLs associated with resistance to ferrous iron toxicity in rice (Oryza sativa L.), using recombinant inbred lines. *Acta agronomica sinica.* 30: 329-333.

Wan, J. L., Zhai, H. Q. and Wan, J. M. (2005). Mapping of QTLs for ferrous iron toxicity tolerance in rice (Oryza sativa L.). *Yi Chuan Xue Bao.* 32: 1156-1166.

WARDA (2008). Annual Report. Responding to the rice crisis. Cotonou, Benin: 60pp

Watanabe, K., Nishiuchi, S., Kulichikhin, K. and Nakazono, M. (2013). Does suberin accumulation in plant roots contribute to waterlogging tolerance? *Frontiers Plant Sci*. 4.

Welch, R. M., Norvell, W. A., Schaefer, S. C. (1993). Induction of iron (III) and copper (II) reduction in pea (*Pisum sativum* L.) roots by Fe and Cu status: Does the root-cell plasmalemma Fe (III)-chelate reductase perform a general role in regulating cation uptake?. *Planta* 190, 555-561.

Widjaja-Adhi, I. P. G., Suriadikarta, D., Sutriadi, M. T., Subiksa, I. B. M., Suastika, I. W. (2000). Pengelolaan, pemanfaatan, dan pengembanganlahan rawa. hal. 127-164. Dalam: Adimihardja, A., L.I. Amien, F. Agus,dan D. Jaenuddin (eds.). Sumberdaya Lahan Indonesia danPengelolaannya. Pusat Penelitian Tanah dan Agroklimat, Bogor.

Widodo, B., Broadley, M. R. and Rose, T. (2010). Response to zinc deficiency of two rice lines with contrasting tolerance is determined by root growth maintenance and organic acid exudation rates, and not by zinc-transporter activity. *New Phytol* 186: 400-414.

Wijerathna, Y. U. C., Dandeniya, W. S. and Mithrasena, Y. J. P. K. (2012). Does oxidizing power of roots affect the iron toxicity tolerance of rice? Book of Abstracts. Peradeniya University Research Sessions, Sri Lanka. 17: 167.

Wu, P., Luo, A., Zhu, J., Yang, J., Huang, N. and Senadhira, D. (1997). Molecular markers linked to genes underlying seedling tolerance for ferrous iron toxicity. *Plant and Soil.* 196: 317-320.

Wu, P., Hu, B.,. Liao, C.Y., Zhu, J. M., Wu, Y. R., Senadhira, D. and Paterson, A. H. (1998). Characterization of tissue tolerance to iron by molecular markers in different lines of rice. *Plant and Soil.* 203: 217-226.

Wu, C., Ye, Z., Li, H., Wu, S., Deng, D., Zhu, Y. and Wong, M. (2012). Do radial oxygen loss and external aeration affect iron plaque formation and arsenic accumulation and speciation in rice? *J. Exp. Bot.*63: 2961-2970.

Wu, L.B., Shhadi, M.Y., Gregorio, G., Matthus, E., Becker, M. and Frei, M. (2014). Genetic and physiological analysis of tolerance to acute iron toxicity in rice. *Rice.* 7: 1-12.

Wu, L. B., Ueda, Y., Lai, S. K. and Frei, M. (2016) Shoot tolerance mechanisms to iron toxicity in rice (Oryza sativa L.). *Plant Cell & Environ.* 40:570-584.

Xing, W., Li, D. and Liu, G. (2010). Antioxidative responses of *Elodea nuttallii* (Planch.) H. St. John to short-term iron exposure. *Plant Physiol. Biochem.* 48: 873-878.

Yamauchi, M. (1989). Rice bronzing in Nigeria caused by nutrient imbalances and its control by potassium sulfate application. *Plant Soil* 117: 275-286.

Yamauchi, M. and Peng, X. X. (1993). Ethylene production in rice bronzing leaves induced by ferrous iron. *Plant Soil.* 149: 227-234.

Yamauchi, M. and Peng, X. X. (1995). Iron toxicity and stress-induced ethylene production in rice leaves. *Plant Soil.* 173: 21-28.

Yamanouchi, M., Yoshida, S. (1981). Physiological mechanisms of rice's tolerance for iron Toxicity. Paper presented at the IRRI Saturday Seminar, June 6, 1981. The International Rice Research Institute, Manila, The Philippines.

Yokosho, K., Yamaji, N., Ueno, D., Mitani, N. and Ma, J. F. (2009). OsFRDL1 is a citrate transporter required for efficient translocation of iron in rice. *Plant Physiol.* 149, 297-305.

Yoshida, S. (1981). Fundamentals of rice crop science. Los Banos, Philippines: *Int. Rice Res. Inst.*

Yoshida, S., Forno, D.A., Cock, J. H. and Gomez, K.A. (1976). Laboratory manual for physiological studies of rice, 3rd edn. *Int Rice Res Inst*, Manila.

Yu, S. M. (1999). Cellular and genetic responses of plants to sugar starvation. *Plant Physiol.* 121: 687-693.

Yukiyoshi, K. and Karahara, I. (2014). Role of ethylene signalling in the formation of constitutive aerenchyma in primary roots of rice. *AoB Plants* 6:43.

Zeng, Z. B. (1994). Precision mapping of quantitative trait loci. *Genetics.* 136: 1457-1468.

APPENDICES

Appendix 1a: Composition of the modified Yoshida nutrient solution (Yoshida, 1976)used for the hydroponic experiments at AfricaRice, Dar es Salaam, Tanzania

	Element	Reagent/Salt	Element final Conc ($mg\ L^{-1}$ or ppm)	Stock solutions (g/L)	Volume of stock (mL/L)	Volume for 1 tray of 10L (ml)
Macro Prepare in 5 separate containers	N	NH_4NO_3	40	91.4	1.25	12.5
	P	KH_2PO_4	10 (8.26 + 1.78)	29 and	1.25	12.5
		K_2HPO_4		8		
	K	K_2SO_4	40	71.4	1.25	12.5
	Ca	$CaCL_2$	40	88.6	1.25	12.5
	Mg	$MgSO_4$	40	158.5	1.25	12.5
Micro Dissolve separately in 100ml, mix all 5 and adjust the volume to 1L	Mn	$MnCl_2.4H_2O$	0.5	1.5	1.25	12.5
	Mo	$(NH_4)_6.MO_7O_2{}_4.4H_2O$	0.05	0.074		
	Zn	$ZnSO_4.7H_2O$	0.01	0.035		
	B	H_3BO_3	0.2	0.934		
	Cu	$CuSO_4.5H_2O$	0.01	0.031		
Separate will be freshly made every week	Fe (control)	$FeSO_4.7H_2O$	2ppm Fe^{2+} or 0.01g/L $FeSO_4.7H2O$	0.1g per tray of 10L – dissolve 0.4g in 200ml		
	Fe (toxicity)	$FeSO_4.7H_2O$	300ppm Fe^{2+} or 1.5g/L $FeSO4.7H2O$	15g per tray of 10L – dissolve 90g in 200ml		

Appendix 1b: Composition of modified Magnavaca nutrient solution (Famoso et al., 2010)used for the hydroponic experiments at IRRI, Los Banos, The Philippines

Element	**Chemical**	**Formula Weight**	**Stock concentration (M)**	**g Chemical / L of stock**
Mg/SO4	MgSO4 7H2O	246.48	0.823	202.853
Ca	CaCl2 2H2O	147.02	1	147.020
P	NaH2PO4 H2O	137.99	0.045	6.210
K/SO4	K2SO4	174.27	0.28075	48.926
NH4/NO3	NH4NO3	80.04	1.428	114.297
HEDTA-Fe	see below			
Micros				
Mn	MnCl2 4H20	197.7	0.01184	2.34
B	H3BO3	61.8	0.03301	2.04
Zn	ZnSO4 7H2O	287.4	0.00306	0.88
Cu	CuSO4 5H2O	249.5	0.0008	0.2
Mo	Na2MoO4 2H2O	241.9	0.00107	0.26
Si	Na2SiO3 9H2O	284.2	0.042224	12
Fe	FeSO4 7H2O	278.01		
Fe-HEDTA	*To make 5 L stock*			
	FeCl3	*270.3*		*4.1628*
	HEDTA	*344.2*		*4.6552*

Appendix 1c: The IRRI index scale for evaluation of Fe^{2+} toxicity (2002)

SCALE (Damaged panicles)	
0	Growth and tillering nearly normal
1	Growth and tillering nearly normal; reddish-brown spots or orange discoloration on tips of older leaves
3	Growth and tillering nearly normal; older leaves reddish-brown, purple, or orange yellow
5	Growth and tillering retarded; many leaves discolored
7	Growth and tillering ceases; most leaves discolored or dead
9	Almost all plants dead or dying