GENETIC VARIATION OF IRON TOXICITY TOLERANCE IN LOWLAND RICE (*Oryza sativa* L.) VARIETIES

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ABSTRACT

Rice (*Oryza sativa L.*) is accepted globally as a major food crop. It is a staple food crop in many countries in Africa. There has been an increasing demand of rice in Africa. Africa consumes 11.6 million tonnes of rice per annum and out of 39 rice producing countries, 21 import 50 to 99 percent of their rice requirements. The inability to reach the yield potential that would sustain Africa's need for rice is due to many biotic and abiotic constraints that rice production faces. In lowland grown rice, one of the abiotic factors hindering rice production is iron toxicity. Excess uptake of ferrous ($Fe^{2+)}$ ions leads to a physiological stress which results into poor production. The best way to control toxicity due to excess ferrous Iron uptake is by the use of tolerant varieties. The current study aimed at selection of varieties tolerant to iron toxicity and assessment of the genetic diversity linked to this trait. In a hydroponic experiment conducted in a screen house at Africa Rice Centre in Dar es Salaam, 32 rice varieties were evaluated for tolerance to iron toxicity. The experiment was laid out in a split plot design with iron concentration as the main plot factor and variety as the sub plot factor. Two levels of iron concentration were used: 2ppm and 300ppm of Fe^{2+} as control and test concentrations, respectively. Traits observed to gauge tolerance were leaf bronzing (an indicator of iron toxicity), plant height, tillering, number of leaves, shoot weight (above ground), root length and root weight. The varieties ARICA8, and CK801 were found to be tolerant due to low bronzing indices, higher shoot weight, more number of leaves and lack of significant variation in morphology between the two Fe treatments except for the plant height. Correlation analysis depicted negative correlation between leaf bronzing and the other traits measured especially shoot biomass. Assessment of genetic diversity was done using 12 SSR rice markers (RM). Out of these, 10 were polymorphic. There was an average 3.9 alleles per loci with an average polymorphic information content of 0.51. The most polymorphic locus was RM 341.

Phylogenetic analysis revealed five clusters with ARICA 8 and CK 801 in two different clusters. In this study, 2 varieties showed tolerance, 22 were partially tolerant and eight were susceptible to iron toxicity. Genetic groups based on the molecular markers were related to rice species rather than iron toxicity tolerance levels.

DECLARATION

I, ZIPPORAH CHUKUPEE PAGE, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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DEDICATION

This work is dedicated to my children and my late husband Jayiah A. Massaquoi, Catherine K. Massaquoi and late Prince V. Massaquoi

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LIST OF ABBREVIATIONS

µl	Microliter
1N HCL	One Normal Hydrogen chloride
1N NaOH	One Normal Sodium hydroxide
ANOVA	Analysis of Variance
BS	Sodium Hydrogen sulfite (Sodium Bisulfite)
CARI	Central Agriculture Research Institute
CEC	Cation Exchange Capacity
DAS	Days after sowing
DNA	Deoxy-Ribonucleic Acid
EDTA	Ethylene di-amine- tetra-acetic Acid
Fe^{2+}	Ferrous Iron
Fe_2O_2	Hematite
Fe^{3+}	Ferric Iron
Fe_3O_4	Magnetite
$FeCO_3$	Siderite
FeooH	Goethite
FeS	Pyrite
IRRI	International Rice Research Institute
K	Potassium
MATAB	Mixed Alkyltrimethyl ammonium Bromide
ml	Milliliter
N	Nitrogen
NERICA	New Rice for Africa

ng	Nanogram
P	Phosphorus
PCR	Polymerase Chain Reaction
PIC	Polymorphism information content
Ppm	Parts per million
QTLs	Quantitative trait loci
ROS	Reactive oxygen species
SSR	Simple sequence Repeat
SUA	Sokoine University of Agriculture
TAE	Tris-Acetate-EDTA
TE	Tris EDTA
WAAPP	West Africa Agriculture Productivity program
WARDA	West Africa Rice Development Association (now AfricaRice)
Zn	Zinc

CHAPTER ONE

1.0 INTRODUCTION

Rice (*Oryza sativa L*) is the worlds' most important food crop, serving as staple food for more than half of the world population (Khush, 2005). It belongs to the family of gramineae and supplies 20 % of the calories consumed by humans. Lowland rice is cultivated on approximately 128 million hectares of irrigated and rain-fed land (Maclean *et al*., 2002). As many as 100 million hectares show some sort of nutritional constraints to rice growth caused by either deficiencies or toxicities (Brady, 1982). Ferrous iron toxicity is among the constraints and primarily affects lowland rice grown on acid flooded soils that are rich in reducible iron (Sahrawat, 2004). Increasing significant occurrence of iron toxicity make it a serious long-term threat to lowland rice production. Large areas of wetland ideally suited for rice production remain underused, especially in Asia, South America, West and Central Africa, because of iron toxicity stress (Ponnamperuma, 1972; Sahrawat, 2004; Fageria *et al*., 2008).

In West Africa, iron toxicity is widespread throughout the humid forest and Savanna zones in about 30 to 40% of the cultivated lowlands (WARDA, 1998). Rice yield losses due to iron toxicity are reportedly ranging from 12 to 100%, depending on the severity of the toxicity and the tolerance of the rice cultivars (Sahrawat and Singh,1995; Audebert and Sahrawat, 2000).

In Liberia, iron toxicity manifestation is mainly a result of heavy rainfalls and river over flows into the valleys. Stagnant inundation of the iron rich soils ultimately lead to the accumulation of high levels of Fe^{2+} in the soil solution. Rice grown in such environments takes up large amounts of ferrous iron, exceeding plant growth's requirements. As a result,

plant metabolism is disturbed and rice yield is dramatically reduced. Data on iron toxicity distribution in Liberia is scanty. According to farmers "lowlands are abandoned and remain uncultivated due to iron toxicity syndrome". Regarding how widespread the constraint was, the government instituted several management options to minimize iron toxicity in the rice fields such as application of limestone fertilizers, ridges, direct planting, and flushing the plot with fresh water but with little success.

1.1 Problem Statement

Rice (*Oryza sativa* L.) is the primary staple food in Liberia (FAO, 2000). Being the most preferred food in Liberia, it is important that its yield is sustainable. However, iron toxicity is a major constraint to rice production. It is a nutritional disorder associated with high level of ferrous iron concentration in the soil and is found only in waterlogged lowlands (Cherif *et al*., 2009). The response of rice to iron toxicity varies among different rice varieties. Some varieties have the mechanism to retain high iron levels in their roots or as oxides in the rhizosphere while other varieties are susceptible to iron toxicity or do not easily adapt to iron toxicity stress (Mandal *et al*., 2004).

Fukuda *et al*. (2012) reported that the surest way to counter iron toxicity is by using tolerant rice varieties. Genetic improvement of iron-toxicity tolerance implies the need of varietal screening to make good use of the existing diversity for iron toxicity tolerance.

1.2 Justification

Lowland soils in Liberia have high concentrations of iron. This is an impediment to rice production in the lowlands. Rice is mostly grown by small holder farmers in Liberia who depend on good harvests as their source of income. Since rice is the staple food in Liberia, a low production due to iron toxicity threatens the country's food security. Cultural

management practices, such as application of limestone fertilizers, ridges, direct planting, though effective in some cases, are either labor intensive or costly. A sustainable approach towards boosting rice yields in iron toxic soils is the use of tolerant varieties. Better understanding the genetic background conferring tolerance to excess Fe^{2+} will make it possible to develop rice varieties with enhanced tolerance to iron toxicity.

1.3 Objectives

1.3.1 Overall objective

To improve yield of lowland rice grown in iron toxic soils of Liberia by using high yielding lowland rice varieties with tolerance to iron toxicity.

1.3.2 Specific objectives

(i) To determine the level of iron-toxicity tolerance in selected lowland rice varieties.

(ii) To assess the genetic diversity of lowland rice varieties in relation with tolerance to iron toxicity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin, Importance and Distribution of Rice

Cultivated rice belongs to the grass species *Oryza sativa* (Asian rice) or *Oryza glaberrima* (African rice). It is a staple food for almost half the world's population (Maclean *et al.,* 2002), and it is grown in a wide range of environments. More than 90 % of global rice production is harvested from irrigated or rainfed lowland rice fields and it accounts for 20–70% of total caloric intake. According to 2012 FAOSTAT data, rice is the most important grain with regard to human nutrition and caloric intake. In Africa, rice is one of the popular foods and it is now the fastest-growing food staple in Africa. The relative growth in demand for rice is faster in Africa than anywhere else in the world (WARDA, 2005). During the past three decades the crop has seen consistent increases in demand and its growing importance is evident in the strategic food security planning policies of many countries. With the exception of a few countries that have attained self-sufficiency in rice production, rice demand exceeds production and large quantities of rice are imported to meet demand at a huge cost in hard currency. Africa consumes a total of 11.6 million tonnes of milled rice per year (FAO, 1996), of which 3.3 million tonnes (33.6 percent) is imported.

2.2 Production Constraints

In Liberia rice is the primary food for 3.5 million people and it plays a major role in the political stability in the country. Notwithstanding, rice production in Liberia faces many constraints and one of the constraints is iron toxicity. Iron toxicity in rice plants occurs when the plants absorb a large amount of iron from the soil. This happens when high concentrations of ferric iron (Fe^{3+}) is reduced into its soluble ferrous form (Fe^{2+}) by

microorganisms under anaerobic conditions (Gross *et al.,* 2003). The critical level for occurrence of iron toxicity in rice plants is 300 ppm Fe in the soil (Veldkamp *et al.,* 1991). The symptoms commonly observed are rusty leaf spots (bronzing), leaf edges are dark brown and the root system poorly developed (Dobbermann and Fairhurst, 2000).

2.3 Iron Toxicity in the Soil

2.3.1 Iron toxicity in the soil and its characteristics

Most soils contain iron. Iron minerals commonly found in soils include goethite (FeOOH), hematite (Fe_2O_4), pyrite (FeS), siderite ($FeCO_3$), and magnetite (Fe_3O_4) (Fageria *et al*., 2003). When parent materials weather, they release significant amounts of minerals in the soil including iron. In reduced or submerged soils, Fe^{2+} concentration can reach high levels. Rice plants growing in such conditions, take up Fe in excess of plant demand, causing toxicity. Iron toxicity occurs in a wide range of soil types but mostly in ultisols, Oxisols and acid sulfate soils. These soils have high ion activity and potential acidity irrespective of organic matter and texture. General characteristics shared by most Fe-toxic soils are high amounts of reducible Fe, low pH, high cation exchange capacity (CEC) and exchangeable potassium content (Ottow *et al*., 1982). Texture, CEC and organic matter content influence the concentration of ferrous iron in soil solution in which iron toxicity occurs (Sahrawat *et al*., 1996). Variations can be observed depending on soil texture and environmental conditions. For instance, iron toxicity was reported to be more severe during the dry season when the vapor pressure deficit is high compared to the wet season (Sahrawat and Singh, 1998; Asch, 2005).

2.3.2 Conditions for Fe^{3+} reduction

The expression of iron toxicity severity in the rice rhizosphere has been related to many soil factors. These included the content and type of clay minerals, the amount of

exchangeable soil Fe^{2+}, the soil pH and the presence of stress factors. The concentration of soil Fe^{2+} is less in clay soils than in sandy soils (Das *et al*., 1997). Meanwhile, clay was found to control the content and distribution of iron in both Alfisols and Vertisols (Rajkumar *et al*., 1997). Clay content may have great potential for Fe^{2+} dynamics as a result of its CEC. High amounts of soluble Fe^{2+} (100–1000 mg L^{-1}) can be found in acid soils (Ponnamperuma, 1972). Acid sulfate soils have Fe^{2+} concentrations of up to 5000 mg kg^{-1} (Van Breemen, 1975).

2.3.3 Distribution of iron in soils

Seasonal changes due to rainfall pattern (rainfed rice) or drainage and irrigation (irrigated rice) affect iron distribution in the soils. Irrespective of these conditions, changes in redox potential (Eh) and Fe concentrations are found on a small scale horizontally in the soil profile and vertically between the bulk soil and the rhizosphere (Howler and Bouldin, 1971). Three soil compartments can be distinguished in paddy soil profiles and they include a thin oxic surface layer, the reduced puddled bulk soil and the oxic rhizosphere soil and rhizoplane (Liesack *et al.,* 2000). The horizontal distribution of Eh and reduced Fe in the profile has been described by Ratering and Schnell (2000). The depth of the oxic surface layer varies between 2 and 10 mm and is partially determined by a nitrate-dependent microbial re-oxidation of Fe^{2+}. The highest Fe^{2+} concentration was found at 2-15 cm soil depth. Concentration of Fe^{2+} declines in deeper layers or below the plough pan. This is where the soil contains less organic matter than in the puddled soil (Revsbech *et al*., 1980). Horizontal variations in Fe^{2+} are linked to the oxic rhizosphere soil, which is the result of oxygen release from rice roots (Yamanouchi *et al*., 1989). Its extent is determined by the formation of aerenchyma (oxidation power of the rice roots) and the root density (Frenzel and Bosse, 1999). The rhizosphere of rice is a potential site of Fe^{2+}oxidation and can also act as a site of iron reduction (Prade, 1987). Anaerobic microorganisms such as

Geobacter, Pseudomonas, Clostridium and Bacillus sp. play a key role in the reduction and mobilization of Fe-oxides (Munch and Ottow, 1977; Trolldenier, 1988). Some fungi are capable of enzymatically reducing Fe (III) oxides (Schwertmann, 1985). The use of oxidized Fe^{3+} as an alternative electron acceptor for respiration requires energy-rich electron donors such as mineralizable organic root exudates and the abundance of iron-reducing microorganisms.

2.4 Iron Toxicity in Rice Plant

2.4.1 Uptake and transport of iron

Rice plants have the tendency of taking up more iron than most of the other plants, and Fe^{2+} is the iron species prevailing in paddy fields. Additionally to the effects of high intracellular Fe concentrations on plant growth, high concentration of Fe^{2+} in the rhizosphere has antagonistic effects in the uptake of many essential nutrients such as xxx and yyy (Fageria *et al*., 2008). Thus, iron toxicity is often described as a multiple nutrient disorder.

Rice plants are able to take up iron from the rhizosphere as Fe^{3+}/phytosiderophore complex but also as Fe^{2+} (Ishimaru *et al.,* 2006). In aerobic environments, where iron is among the most limiting nutrients for plant growth primarily as a result of the low solubility of the oxidized ferric form (Zuo and Zhang, 2011; Samaranayake *et al*., 2012), iron is absorbed as Fe^{3+}/phytosiderophore. Iron in the form of Fe $(OH)_3$ is solubilized by phytosiderophores from the mugineic acid (MA) family to form a Fe(III)-MA complex. The Fe(III)–MA complex is then transported through the root membrane via a transporter from the Yellow Stripe (YS) family (Inoue *et al.*, 2009). YS Fe transporter was first isolated in maize and named YS1 (Curie *et al*., 2001). Within the rice cells, Fe may move in the form of Fe-citrate, Fe^{2+}-nicotianamine (NA) and Fe^{3+}- 2'-deoxymugineic acid

(DMA) (Koike *et al*., 2004). These complexes are readily taken up by specific receptors which will ensure the passage of Fe from one cellular compartment to another (Bashir *et al*., 2006). Fe translocation from the roots to the shoots and its distribution to organs are done through the xylem and phloem saps in the form of Fe/chelator complex (Hell and Stephan, 2003). Fe is bound to chelators (citrate, NA or DMA) to prevent formation of free radicals (Kim and Guerinot, 2007).

Fe^{+} uptake and translocation in rice plant is illustrated in Fig. 1.

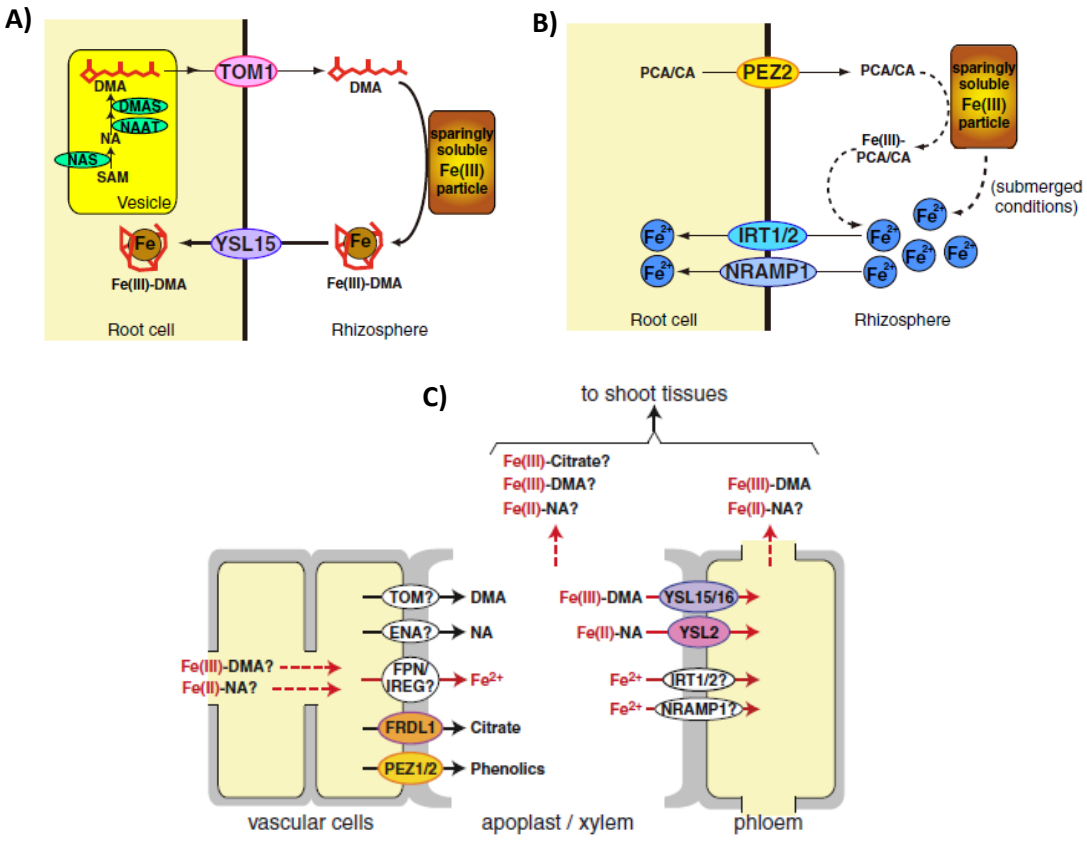

Figure 1: Iron uptake and translocation in rice root. A) and B) Iron uptake mechanisms through complexation of Fe and direct uptake of Fe respectively. C) Iron translocation in vascular cells of rice root towards the shoot. Ovals represent transporters and iron flow is depicted in red (Kobayashi *et al*., 2014)

2.4.2 Symptoms of iron toxicity effects

Appearance of iron toxicity symptoms in rice is a result of excessive Fe^{2+} uptake by rice roots and its translocation into leaves. Excess Fe^{2+} uptake elevates production of toxic oxygen radicals that can damage cell structural components and impair physiological processes (Becker and Asch, 2005). For instance, oxygen radicals can destroy membrane lipids and DNA (Fukuda *et al*., 2012). When plants absorb high amounts of iron, discoloration of leaves can be observed. This phenomenon is called leaf bronzing (Wu *et al.,* 1998) and may be due to oxidized polyphenols (Peng and Yamauchi, 1993). Leaf bronzing begins as small brown spots on the tip of lower leaves and extends towards the leaf base (Wu *et al*., 2014). The spots coalesce and the lower leaves turn dark purplish brown (Wan *et al*., 2005) and eventually die. Other symptoms depend on the stage when leaf bronzing occurs. If bronzing occurs at the seedling stage, the plants remain stunted with limited tillering (Abraham and Pandey, 1989). During the vegetative stage, the symptoms are reduced plant height and dry-matter accumulation (Abu *et al.,* 1989), with the shoot being more affected than the root biomass (Fageria, 1988).

2.5 Mechanisms of Tolerance to Iron Toxicity

2.5.1 Physiological avoidance and exclusion

Tolerance to iron toxicity in rice is governed by age, nutritional status and chemical environment. The physiological status of a rice plant under submerged soil condition greatly modifies its ability to tolerate high iron concentration in the soil solution. In the presence of P, K, Zn and N, low effects of iron toxicity can be observed (Audebert and Sahrawat, 2000). Rice roots play three important functions to counter iron toxicity. These include oxidation of iron in the rhizosphere to keep iron concentration low in the growth media. In this process, molecular oxygen is transferred from the shoots to the roots through air chambers and aerenchyma and then diffused into the medium. This makes the

rhizosphere more oxidized than the bulk growing soil. The oxidative power of the roots greatly influences the chemosphere around it and leads to the oxidation of ferrous iron (Fe) in the soil solution into its ferric iron (Fe) (Mandal *et al*., 2004). Thus, a diminished ability of the rice roots to oxidize Fe into Fe results in a higher uptake of iron (Sahrawat, 2000). The oxidizing power of the rice roots is greater at the growing points and at the elongating parts than at the basal parts of the roots (Yamanouchi and Yoshida, 1981).

This process is known as exclusion and it leads to a considerable Fe accumulation and the formation of iron plaque around rice roots (Kirk *et al*., 1990). Roots can also retain Fe in the root tissues after Fe is taken up from the rhizosphere. This in turn decreases the translocation of iron from the root to the shoot (Tadano, 1975). According to Audebert and Sahrawat (2000), iron toxicity-tolerant rice cultivars absorb less iron or translocate less iron from the roots to the leaves. Both processes are called physiological avoidance. Iron-tolerant rice cultivars accumulate less iron in the photo- synthesizing leaves and maintain superior photosynthetic potential in the presence of absorbed iron in the leaves (Audebert and Sahrawat, 2000). Since plant growth depends on dry matter production by leaves (Jiang *et al*., 2004), rice cultivars tolerant to iron toxicity are expected to have higher shoot biomass than susceptible ones.

2.5.2 Inclusion

In other iron-tolerant varieties, relatively high levels of Fe can be found in the tissues without severe toxicity symptoms. These varieties are thought to have good adaptation mechanisms to increasing reactive oxygen species (ROS) brought about by Fenton reactions as a result of iron toxicity. This is achieved by the use of antioxidants such as ascorbic acid and reduced glutathione (Gallie, 2013) but also by the activation of enzymes such as superoxide dismutase, peroxidase and catalase which protect plants from ROS

damage (Fang *et al*., 2001). Another mechanism is storage of Fe in non-reactive forms such as Fe-ferritin or Fe-phytate. Storage in the apoplast and vacuole, and detoxification of Fe-induced reactive oxygen species (ROS) by antioxidant enzymes are the mostly reported tolerance mechanisms (Dufey *et al*., 2009).

2.6 Varietal Selection and Screening for Resistance to Fe toxicity

Fukuda *et al.* (2012) reported that the surest way to counter iron toxicity is by using tolerant rice varieties. Many cultivars are hypothesized to employ tolerance rather than avoidance or exclusion mechanisms (Becker and Asch 2005; Yamanouchi and Yoshida 1981). These mechanisms could be important selection criteria of tolerant rice genotypes. On the other hand, successful crop improvement depends on genetic variability that arises from genetic diversity (Rana and Bhat, 2004). Lack of genetic diversity may limit breeding progress and gain from selection (Cornelius and Sneller, 2002). Breeders have developed a wide array of cultivars with various degrees of adaptation, using traditional breeding methods (Akbar *et al*., 1986; Gunawardena *et al*., 1982) and possibly marker-assisted selection breeding (Wan *et al*., 2003). Several QTLs were reported for Fe toxicity tolerance in rice (Wan *et al.*, 2003; Dufey *et al.*, 2009; Fukuda *et al.*, 2012; Wu *et al.*, 2014). Traits found to be closely linked to iron toxicity tolerance in these loci include leaf bronzing, root length, root weight, plant height, tillering and number of leaves. The use of molecular markers associated with these QTLs could significantly facilitate the characterization of rice germplasm for iron toxicity tolerance. Simple sequence repeats (SSR) are among the most widely-used DNA marker types to characterize germplasm collections of crops (Van Inghelandt *et al*., 2010). SSRs have been effectively used to identify genetic variation among rice cultivars (Caicedo *et al*., 2007) and possess considerable potential for genetic fingerprinting.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Location and Duration of Study

A hydroponic culture screening experiment was conducted in a screen house at the Africa Rice Centre in Mikocheni, Dar es Salaam, Tanzania. Exact geographical coordinates are as follows: latitude 6° 45' 49.4748" S, longitude 39° 14' 43.2312" E and altitude 17 m above sea level. The experiment was conducted from December 2014 to April 2015 followed by lab work at the same location.

3.2 Plant Materials

Thirty two rice varieties, including tolerant and susceptible checks were screened for tolerance to iron toxicity. The tolerant checks were SUAKOKO-8 and WITA-4, while the susceptible check was IR64. Varieties were provided by the Africa Rice Center. These varieties are suited to lowland ecology and are adapted to different countries of sub-Saharan Africa. The rice varieties are listed according to their species, origin, names and code as shown in Table 1.

Table 1: Rice cultivars used in the experiment

No.	Variety Name	Code Exp.	Species	Origin
1	CK90	V1	*O. sativa (indica)*	Guinea
2	72 – 5	V2	*O. glaberrima*	Liberia
3	SUPA	V3	*O. sativa (indica)*	Tanzania
4	ARICA 1	V4	*O. sativa (indica)*	AfricaRice
5	SARO 5	V5	*O. sativa (indica)*	Tanzania
6	IR841	V6	*O. sativa (indica)*	IRRI
7	WITA4	V7	*O. sativa (indica)*	AfricaRice
8	TXD88	V8	*O. sativa (indica)*	Tanzania
9	SAHEL 201	V9	*O. sativa (indica)*	Senegal
10	SUAKOKO8	V10	*O. sativa (indica)*	Liberia
11	ARICA 3	V11	*O. sativa (indica)*	AfricaRice
12	NERICA-L19	V12	*Interspecific O. sativa x O. glaberrima*	AfricaRice
13	TOG 16771	V13	*O. glaberrima*	Guinea
14	ARICA 6	V14	*O. sativa (indica)*	AfricaRice
15	IR64	V15	*O. sativa (indica)*	IRRI
16	NERICA-L23	V16	*Interspecific O. sativa x O. glaberrima*	AfricaRice
17	TOG 6241	V17	*O. glaberrima*	Liberia
18	ARICA 8	V18	*O. sativa (indica)*	AfricaRice
19	KALAMATA	V19	*O. sativa (indica)*	Tanzania
20	JASMINE 85	V20	*O. sativa (indica)*	AfricaRice
21	FOFIFA 172	V21	*O. sativa (japonica)*	Madagascar
22	SHAKA 102	V22	*O. sativa (japonica)*	Guinea
23	CK 801	V23	*O. sativa (indica)*	Guinea
24	TOG 6635	V24	*O. glaberrima*	Liberia
25	ORYLUX 6	V25	*O. sativa (indica)*	AfricaRice
26	ARICA 2	V26	*O. sativa (indica)*	AfricaRice
27	CK73	V27	*O. sativ (indica)*	Guinea
28	YUNKENG	V28	*O. sativa (japonica)*	Rwanda
29	ORYLUX 4	V29	*O. sativa (indica)*	AfricaRice
30	ARICA 7	V30	*O. sativa (indica)*	AfricaRice
31	YUN YINE	V31	*O. sativa (japonica)*	Rwanda
32	BOTRY	V32	*O. sativa (japonica)*	Madagascar

3.3 Selection of Tolerant Varieties

3.3.1 Experimental setup

To distinguish rice varieties tolerant to iron toxicity from the susceptible ones, the seedlings were treated with toxic levels of iron in a hydroponic experiment as described by Wan *et al.* (2003). Seeds were sown into a perforated polystyrene plate floating on the surface of nutrient solution contained in 14 L-plastic trays. For each variety, 6 plants were grown at a rate of two plants per hole. Yoshida standard rice nutrient solution (Yoshida *et al.,* 1976) was used as the normal treatment for plant growth. For Fe^{2+} stress treatment, 300 ppm Fe^{2+} was added as $FeSO_4$ to the standard nutrient solution. To maintain acidic conditions, the pH of the solution was adjusted once a week at 5.6-5-7 using 1N NaOH or 1N HCl at the time of solution change (every week). Fe stress treatment was maintained for 3 weeks. The standard nutrient solution and the trays layout are described in Table 2 and Fig. 2 respectively.

Table 2: Composition of Yoshida standard rice nutrient solution used in the hydroponic experiment

	Element	Reagent/salt	Element final cont (mg L^{-2} or ppm)	Stock solutions (g/L)	Volume stock (mg/L)	Volume for 1 tray of 0L (ml)
Macro prepare in 5 separate containers	N	NH_4NO_2	40	91.4	1.25	12.5
	P	KH_2PO_4	10(8.26+1.78)	29 and	1.25	12.5
		KH_2PO_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 100 ml, mix all 5 and adjust the volume to 1L	Mn	$MnCl_24H_2O$	0.5	1.5	1.25	12.5
	Mo	$(NH4)_2$, MO_7, O_2, $4H_2O$	0.05	0.074		
	Zn	$ZnSO_4$, $7H_2O$	0.01	0.035		
	B	H_2BO_2	0.2	0.934		
	Cu	$CuSO_4$, $5H_2O$	0.01	0.031		
Separate will be freshly made every week	Fe (control)	Fe, SO_4, $7H_2O$	2 ppmFe^{2+} or 0.01g/l $FeSO4$, $7H_2O$	0.1g per tray of 10L –dissolve 0.4g in 200 ml		
	Fe (toxicity)	Fe, SO_4, $7H_2O$	300 ppmFe^{2+} or 1.5g/l $FeSO4$, $7H_2O$	15g per tray of 10L –dissolve 90g in 200 ml		

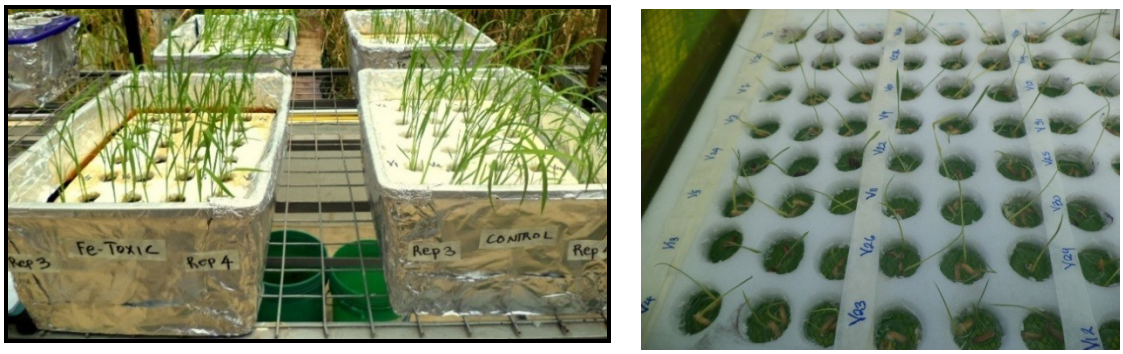

Figure 2: The experimental setup in a screen house at the Africa Rice Centre Mikocheni, Tanzania

3.3.2 Experimental design

The experiment was conducted in a randomized complete block design arranged in a split plot manner with four replications. The main plot had two levels of iron concentration and thirty two (32) varieties were allocated to the sub-plots. Each iron concentration (main plot) was assigned to one tray (28 cm x 31 cm) containing nutrient solution with 2 or 300 ppm Fe^{2+}. All 32 varieties were planted on the same polystyrene float (26 cm x 29 cm) with a spacing of 1.5 cm x 1.5 cm between plants and between varieties. Each variety (sub-plot) occupied 3 holes on the float as shown in Fig 2.The experimental layout showing the randomization of iron concentrations and varieties is described in appendix 1.

3.3.3 Data collection

3.3.3.1 Environmental Conditions

The experiment was conducted in a screen house at ambient conditions at Africa Rice Centre in Mikocheni. Dar es Salaam, Tanzania. The temperature and relative humidity were recorded using a data logger (Dickson Data Logger TP125) between December 2014 and April 2015. The variables collected in the experiment were leaf bronzing, plant height, number of leaves, number tillers, dry shoot weight, root length dry and root weight.

3.3.3.2 Scoring of Leaf Bronzing

The leaf bronzing score was recorded every week starting from the first observation (10 days after imposing the stress) in reference to the scale of the Standard Evaluation system for Rice (IRRI, 2002). This scale varies from score 0 to 9 as described in Table 3. This was adopted for iron toxicity severity.

Table 3: Standard evaluation system for rice (IRRI, 2002)

Score	Symptoms
0.	Growth and tillering nearly normal
1.	Growth and tillering nearly normal reddish-brown spots 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 ore dead
9.	Almost all plans dead or dying

3.3.3.3 Number of tillers, number of leaves, and root length

Number of tillers and leaves were collected by counting on each plant and data were recorded. Plant height was measured using a steel ruler from the ground level to the tip of the longest leaf. Root length was measured using the same steel ruler.

3.3.3.4 Dry weight of shoot and root

All six plants per variety were harvested fresh at the end of the stress period, at 5 weeks after sowing to determine dry matter accumulation. Separate root and shoot samples were wrapped in an aluminum foil and oven dried at 72^{o}C to constant weight. Then dry weights were determined.

To determine trait variation between Fe stress and control, relative performances were calculated follows:

$$\text{Relative reduction} = \frac{DW_{\text{control plant}} - DW_{\text{treated plant}}}{DW_{\text{control plant}}} \text{ x } 100$$

This was calculated for all the parameters except leaf bronzing which was observed only on Fe-treated plants.

3.3.4 Data analysis

Data analysis was done using Genstat statistical package (15th edition 2015). ANOVA was performed to analyze the experimental data on plant height, number of tillers and leaves, shoot weight, root weight, root length and leaf bronzing. Means separation was done using Duncan Multiple Range test. Correlation analysis was further performed to establish relationship between leaf bronzing and other traits (plant height, tillers, leaves, shoot weight, root weight, root length).

3.4 Genetic Diversity of the Collection Tested

3.4.1 DNA extraction

Young leaf samples were collected, wrapped in aluminum foil and stored at -20°C until further use. Genomic DNA was extracted from the leaf samples following the protocol described by Romero *et al.* (2014). Basically, the rice leaves (~100 mg) were cut into small pieces in 2ml tubes properly labelled and containing 6 2.8mm-beads and a small pinch of 1.4mm beads. This was followed by addition of 600 µL of extraction buffer composed of Tris100 mM pH 8, NaCl 1.4 M, EDTA 20 mM pH 8, MATAB 3 % and BS 0.5 % preheated at 74°C. The samples were ground using Precellys 24 grinder (5000trm – 2x20 sec), until well ground. The tubes containing ground samples were then incubated at 74°C in a water bath for at least 30 min and the samples cooled at room temperature

before adding 500 µl of chloroform /isoamylalcohol mix (24:1). The solutions were gently mixed by inversion for 2 min and centrifuged at 12000g at room temperature for 15 min. The supernatant was transferred into new tubes (~500 µl) and 300 µl of cold isopropanol (stored at -20°C) was added. Afterwards, solutions were gently mixed by inversion, and kept at a temperature of -20°C for at least 15 min. After centrifugation at 12000g for 15 min, the supernatants were carefully removed and the pellets were washed with 100 µl of 70% cold ethanol (stored at -20°C). Thereafter the supernatant was discarded after a centrifugation at 12000g for 10 min and the resulting DNA pellets were dried at room temperature. The DNA concentrations were determined using a Nanodrop 2000 C (Thermo scientific USA) and the samples were diluted to 25 ng/µL before using.

3.4.2 Polymerase Chain Reaction (PCR)

DNA amplification was carried out using 12 SSR markers linked to tolerance to iron toxicity (Table 4). The PCR cocktail ingredients included 50 ng of each DNA sample, 10× PCR buffer (2µL) supplemented with $MgCl_2$, 1µL dNTP's at 10 mM, 1µL of each forward and reverse primers at 10µM and 0.2µL of Taq polymerase at 5U/µL. One reaction mixture for each marker had a total volume of 20 µL. The PCR tubes containing the reaction mix were transferred into a thermo cycler (Biometra) where the DNA was amplified using the following PCR profile: 94°C for 5 min (denaturation), followed by 35 cycles of 94°C for 30sec, 55°C for 45sec (annealing), 72°C for 1 min (elongation) and a final extension at 72°C for 5 min. The PCR products were held in the fridge until further use.

Table 4: Sequences of primers used for the diversity analysis

Name	Forward primer	Reverse primer	References
RM443	GATGGTTTTCATCGGCTACG	AGTCCCAGAATGTCGTTTCG	Dufey *et al*., 2014
RM403	GCTGTGCATGCAAGTTCATG	ATGGTCCTCATGTTCATGGC	Dufey *et al*., 2012
RM561	GAGCTGTTTTGGACTACGGC	GAGTAGCTTTCTCCCACCCC	Dufey *et al*., 2012
RM333	GTACGACTACGAGTGTCACCAA	GTCTTCGCGATCACTCGC	Onaga *et al*., 2013
RM11488	CAGACCAAGGAGGAGAATGAAGG	AGTTGTTGCCTGCACTACACAGC	Dufey *et al*., 2014
RM11514	CTTGCTCTGATGCTGACAGATAAACC	GGAGTCCATGTCATATGTGCTTTCC	Dufey *et al*., 2014
RM11522	TAACTGCAGTGCTCAACAAAGG	CTAGGTACCGGATTAAGATTCACC	Dufey *et al*., 2014
RM11529	ATGGATCGCGACATCCACTAGC	GGCAAAGTTGACAACACGTACCC	Dufey *et al*., 2014
RM341	CAAGAAACCTCAATCCGAGC	CTCCTCCCGATCCCAATC	Onaga *et al*., 2012
RM221	ACATGTCAGCATGCCACATC	TGCAAGAATCTGACCCGG	Shimizu *et al*., 2009
RM6	GTCCCCTCCACCCAATTC	TCGTCTACTGTTGGCTGCAC	Wan *et al*., 2005
RM429	TCCCTCCAGCAATGTCTTTC	CCTTCATCTTGCTTTCCACC	Dufey *et al*., 2012

3.4.3 Agarose gel electrophoresis

In order to visualize PCR products and determine allelic composition, the PCR products were subjected to electrophoresis in a 3% Agarose gel stained with a substitute of Ethidium Bromide, Nancy 520 (Sigma). The gel was prepared by dissolving 9g of Agarose powder in 300 ml of 1× Tris Acetate EDTA (TAE) buffer. The gel stain was added to the Agarose prior to gel casting. After polymerization, the gel was immersed in an electrophoresis tank filled with 1× TAE buffer and aliquots of 10µL of the PCR products mixed with loading dye were loaded. The samples were allowed to run in the gel at 200V for 1h. The gel was then visualized in a UV – trans-illuminator to show the DNA bands.

3.4.4 Data analysis

Two markers that did not show polymorphism and four varieties with a high number of missing data were excluded from the analysis. Hence the following analyses were carried out using data from 12 markers and 28 varieties. Pair wise comparison of genotypes, based on allelic data was used to generate dissimilarity coefficients by Darwin V.6. These coefficients were used to generate a dendogram by the unweighted paired group method with arithmetic means (UPGMA). Polymorphism information content (PIC) was calculated using Power marker V.3.25.

CHAPTER FOUR

4.0 RESULTS

4.1 Environmental Conditions During the Experiment

The hydroponic culture experiment was conducted in a screen house for five weeks including three weeks of stress to validate and select varieties which are tolerant to iron toxicity. Environmental conditions during this period indicated that daily maximum temperature ranged from 45.6^{o}C to 49.1^{o}C while the minimum daily temperature was between 22.9°C and 32.5°C. The highest temperature was recorded in March while the minimum temperature was in December 2014 (Fig. 3). The average minimum and maximum temperature during the growing season were 34.4°C and 39.4°C which was favorable for rice production.

The maximum daily relative humidity (RH) ranged from 89.7% to 99.5% while the minimum daily relative humidity ranged from 26.6% to 28.5%. The highest and lowest relative humidity were both observed in March suggesting large variations of RH during this month. The average minimum and maximum relative humidity during the experimental period was 61.3% and 77.9% which was favorable for rice production described in Fig 3.

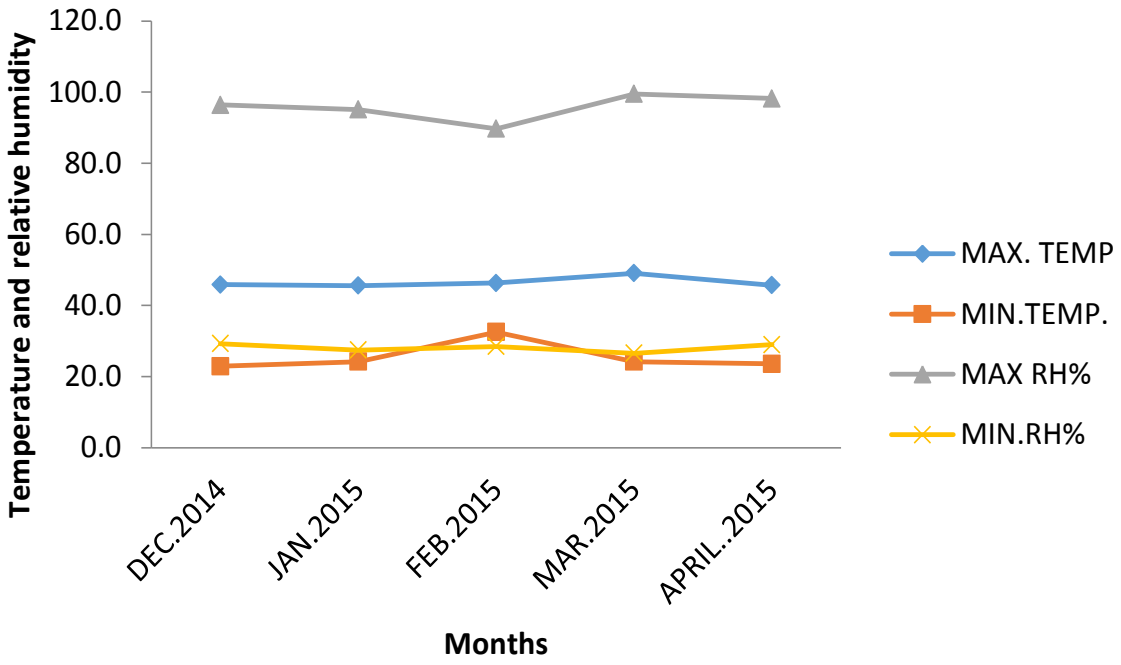

Figure 3: Maximum and minimum temperature and relative humidity during the experimental period

4.2 Selection of Tolerant Varieties Based on Relative Performances under Fe Stress

4.2.1 Effects of Fe^{2+} stress on the varieties tested

The progress of Fe^{2+} stress on the varieties tested at different stages is shown in Fig 4. At week one, the reduction of plant growth was visible on leaf number and leaf width. At week two, leaf bronzing symptoms appeared on Fe-treated plants and plant height and shoot biomass were strongly reduced. The longer the duration of Fe^{2+} treatment on the plants, the more intense the effect on plant growth was. Thus at week three, leaf bronzing increased and many varieties stopped growing.

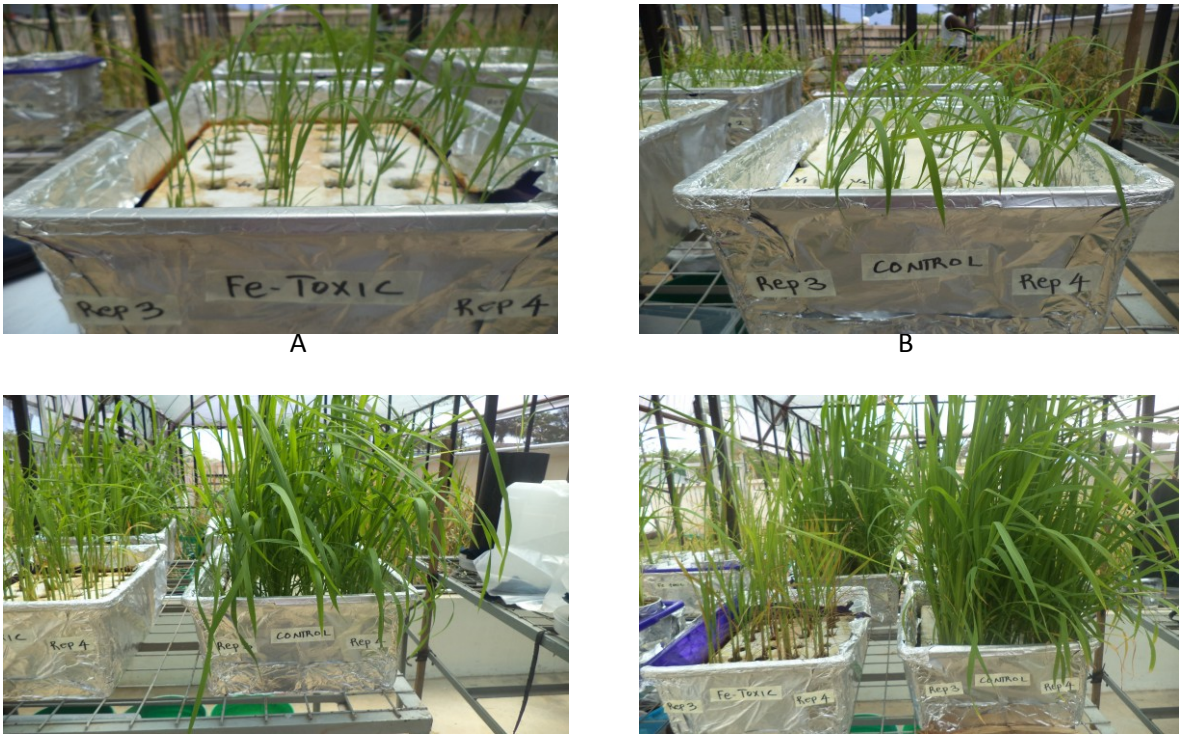

Figure 4: General aspect of rice plants growing in control and Fe^{2+} treatment in the screen house. A) Fe-treated plants after one week of stress. B) Control plants during the same period. C) Fe-treated and control plants at week two. D) Fe-treated and control plants at week three.

4.2.2 Analysis of variance (ANOVA) for 32 rice varieties

Analysis of variance was conducted to estimate the effects of variety (Var), Fe concentration and the interaction between variety and Fe concentration (Var x Fe Conc) on the variation of the different traits measured (Table 5). The results indicated that Fe concentration showed highly significant and very highly significant difference among the traits measured. Similarly very highly significant differences among varieties were revealed for all traits. On the other hand, non-significant differences were observed for the Fe concentration x variety interaction for plant height and root length. Tiller number and leaf bronzing showed very highly significant differences for the interaction while leaf number and shoot dry weight showed highly significant differences.

Table 5: Summary of ANOVA table showing variation in mean squares and F probability among varieties

Source of variation	Df	Plant Height (cm)	Leaf Number	Tiller Number	Root length (cm)	Root Dry Weight (g)	Shoot Dry Weight (g)	Bronzing
Rep	3	766.25***	2.16 ns	0.40 ns	52.49**	0.054*	1.78***	12.58***
Fe Conc.	1	24391.79***	736.10***	28.05***	2846.69***	1.49**	27.78***	833.49***
Var.	31	1014.59***	9.71***	0.69***	3.28***	0.05***	0.29***	2.20***
Fe Conc. *Var	31	43.70 ns	7.9**	0.59***	1.17 ns	0.03*	0..18**	2.20***
Error (Var)	3.73							
Total	**250**							

Where: ***= P<0.001, ** = P<0.01, * = P<0.05 significant levels at indicated P and ns = not significant level

4.2.3 Variation of plant height, leaf number, tiller number and root length among the varieties and Fe treatments

After three weeks of exposure to Fe stress (300 ppm Fe^{2+}), plant height of tested varieties for the control treatment ranged from 47.5 cm (Saro 5) to 90.5 cm (Shaka102) with a mean of 68.6 cm while for the Fe^{2+} treatment, average plant height was 48.9 cm with the highest height attained by CK 90 (68.67 cm) and the lowest by Saro5 (34.2 cm).

The range of leaf number under control treatment was between 6.5 (CK 73) and 12.6 (IR 64) with a mean of 9.9 while for Fe treatment the range was between 4.5 (Botry) and 7.3 (Shaka 102) with a mean of 6.1. Tiller number for control plants ranged from 0.0 (CK 73) to 1.9 (Orylux4) with a mean of 0.8 while under Fe^{2+} treatment, the range was from 0.0 (IR64) to 0.5 (Jasmine 85) with a mean of 0.1. Root length under control treatment ranged from 15 cm (Jasmine 85) to 26.5 cm (Tog 16771) with a mean of 19.3 cm. In the Fe treatment the range was from 9.2 cm (Kalamata) to 17.63 cm (72-5) with a mean of 12.6 (Table 6).

Table 6: Effect of Fe^{2+} stress on variables plant height, tiller number, leaf number and root length at the third weeks of Fe treatment

No.	Varieties	Plant height (cm)		Leaf number		Tiller number		Root length (cm)	
		Control	Fe^{2+}	Control	Fe^{2+}	control	Fe^{2+}	Control	Fe^{2+}
1	SARO5	47.5a	34.2a-c	10.7a-d	7.0d	1.1a-h	0.4b-f	18.1a-c	12.1a-f
2	IR64	53.6a-b	36.1a-d	12.6de	6.3cd	1.6g-h	0.0a	16.4ab	10.1ab
3	IR841	55.8a-c	36.6a-d	10.9a-d	6.6d	1.2c-h	0.5f	19.4a-c	12.1a-f
4	TXD88	53.3a-b	36.7a-d	12.5de	6.0b-d	1.2d-h	0.0a	20.4a-d	12.9a-g
5	SAHEL201	57.6a-d	37.5a-c	11.5c-e	6.4d	1.4e-h	0.1a-c	22c-e	11.8a-f
6	YUNKENG	52.9a-b	38.5a-e	7.2a-c	6.0c-d	0.0a	0.2a-f	18.2a-c	13.3a-h
7	NL23	53.8a-b	39.2a-f	7.6a-c	5.6b-d	0.2a-d	0.1a-c	18.7a-c	12.4a-f
8	ARICA6	62.3b-f	39.9a-f	11.2b-e	5.5b-d	0.8a-h	0.0ab	16.5a-b	9.6ab
9	ARICA7	54.8a-b	40.3a-f	11.3c-e	6.4d	1.3d-h	0.0ab	15.6a	12.2a-f
10	ARICA1	57a-d	41.0a-f	9.4a-d	7.1d	1.2b-h	0.2a-f	15.2a	11.0a-d
11	ARICA2	63.1b-f	41.6a-f	7.8a-c	5.5b-d	0.2a-d	0.0ab	15.2a	12.9a-g
12	ORYLUX4	64.6b-f	42.9a-g	15.2d	6.7d	1.9h	0.1a-c	17.7a-c	10.3ab
13	WITA4	58.7a-e	43.2a-h	9.5a-d	6.4d	0.5a-g	0.2a-f	19.9a-c	14.2b-n
14	YUNYINE	62b-f	43.6a-h	7.9a-c	5.2b-d	0.0a	0,0a-d	15.1a	10.8a-d
15	ARICA3	63.7b-f	43.8a-h	10.8a-d	5.2b-d	1.1b-h	0.1a-c	19.3a-c	12.2a-f
16	JASMINE85	56.8a-d	45.4b-h	9.6a-d	7.0a-h	0.8a-h	0.5f	15.0a	10.4ab
17	ARICA8	72.2f-h	49.2b-i	10.7a-d	6.2c-d	1.2c-h	0.0a-c	18.5a-c	11.2a-d
18	ORYLUX6	76.7g-i	50.0c-i	10.7a-d	6.1c-d	0.9a-h	0.0a-d	18.5a-c	10.7a-c
19	CK73	68.6d-g	50.8a	6.5a	2.3a	0.0a	0.0a	17.5a-c	11.5a-d
20	FOFIFA172	67.4c-g	50.9c-j	7.8a-c	5.5b-c	0.5a-f	0.0a-d	22.0c-e	15.7e-h
21	TOG16771	70.5e-g	51.8c-j	11.3b-e	5.8b-d	1.5f-h	0.2a-f	26.5u	13e-g
22	72-5	67.7c-g	53.3d-j	11.1b-e	6.0b-d	1a-h	0.0ab	22.9c-e	17.6h
23	KALAMATA	84.4ij	55.8e-j	7.8a-c	5.8b-d	0.3a-e	0.1a-e	21.8b-e	9.2a
24	SUAKOKO8	82.1h-j	56.5f-j	9.3a-c	6.2c-d	0.6a-g	0.0a-d	20.1a-d	11.4a-e
25	NL19	86.1ab	56.9f-j	8.3a-d	6.3cd	0.2a-d	0.0a-d	21.0o-u	15.0c-h
26	BOTRY	85.7i-j	61.0g-j	7.9a-c	2.2a	0.4a-f	0.0ab	21.3b-d	13.1a-g
27	CK801	81.5h-j	61.2g-j	7.4a-c	6.2c-d	0.1a-c	0.0a-d	20.3a-d	16.9gh
28	TOG6635	82.5h-j	61.6h-j	10.0a-d	6.2c-d	1.2c-h	0.0ab	21.2b-d	16.1f-h
29	SUPA	90.3j	63.1b-h	7.3a-c	4.4bc	0.1ab	0.0a	21.2b-d	9.3a
30	TOG6241	83.h-j	65.06b-i	9.8a-d	4.1b	0.9a-h	0.1a-c	25.2de	15.9e-h
31	CK90	87.1ij	68.j	6.8ab	6.6d	0.00a	0.0a-d	19.6a-c	15.2d-h
32	SHAKA102	90.5j	66.5ij	8.4a-d	7.3a-i	0.5a-f	0.4b-f	18.9a-c	13.7a-h
	Mean	68.61	48.9	9.6	6.13	0.8	0.1	19.37	12.66
	SE	7.1	10.80	2.55	1.12	0.64	0.24	3.11	2.61
	CV%	10.5	23.4	26.6	19.0	82.1	232.5	16.0	20.7

Numbers followed by the same letter (s) in columns are not significantly different at $P \leq 0.05$ using Duncan Multiple Range test

4.2.4 Variation of dry shoot weight and root weight among the varieties and Fe treatments

The study found that there were significant differences among the varieties. Shoot dry weight under control treatment ranged from 0.41 g (ARICA 7) to 1.5 g (NL 19) with a mean of 0.92 g while for Fe treatment shoot weight ranged from 0.12 g (CK 73) to 0.41 g (ARICA 8). On the other hand, root dry weight for control ranged from 0.6 g (ARICA 1) to 0.48 g (Orylux 4) with a mean of 0.26 g whereas, for Fe treatment, the range was from 0.4 g (CK 73) to 0.16 g (ARICA 8) with a mean of 0.11 g (Table 7). On average, 28.7 % reduction was observed in plant height between control and Fe stress. This was the greatest reduction since leaf number was reduced by only 0.33 %, shoot weight by 71.7 %, root weight by 61 % and root length decreased by 34.6 %.

Table 7: Effect of Fe^{2+} on shoot dry weight and root dry weight of varieties exposed to control Fe and 300ppm Fe^{2+} for three weeks

		Shoot dry weight (g)		Root dry weight (g)	
No.	**Varieties**	**Control**	**Fe^{2+}**	**Control**	**Fe^{2+}**
1	SARO5	1.10b-k	0.25a-c	0.45h-k	0.09ab
2	IR64	0.52a-b	0.19ab	0.11a-c	0.07ab
3	IR841	0.85a-j	0.25a-c	0.18a-f	0.08ab
4	TXD88	1.06a-k	0.22a-c	0.21a-g	0.08ab
5	SAHEL201	0.49a-b	0.19ab	0.15a-e	0.09ab
6	YUNKENG	0.65a-g	0.22a-c	0.07ab	0.08ab
7	NL23	0.58a-f	0.15a	0.09ab	0.06a
8	ARICA6	0.96a-k	0.22a-c	0.16a-f	0.09ab
9	ARICA7	0.41a	0.21a-c	0.07ab	0.12a-c
10	ARICA1	0.62a-g	0.26a-c	0.06a	0.08ab
11	ARICA2	0.56a-e	0.25a-c	0.11a-c	0.09ab
12	ORYLUX4	0.95a-k	0.23a-c	0.48j-k	0.07ab
13	WITA4	0.65a-g	0.22a-c	0.13a-d	0.13a-d
14	YUNYINE	0.44ab	0.16ab	0.11a-c	0.05a
15	ARICA3	0.94a-k	0.21a-c	0.20a-f	0.08ab
16	JASMINE85	0.72a-h	0.24a-c	0.17z-f	0.08ab
17	ARICA8	1.51jk	0.41a-f	0.36e-k	0.16a-f
18	ORYLUX6	1.15c-k	0.19ab	0.23a-h	0.06a
19	CK73	0.54a-d	0.12a	0.11a-c	0.04a
20	FOFIFA172	0.92a-k	0.39a-f	0.15a-e	0.11a-c
21	TOG16771	1.23f-k	0.31a-e	0.44h-k	0.13z-d
22	72-5	0.51a-c	0.29a-d	0.25i-a	0.11ab
23	KALAMATA	1.16d-k	0.25a-c	0.24a-h	0.14a-e
24	SUAKOKO8	0.69a-h	0.23a-c	0.19a-f	0.15a-e
25	NL19	1.56k	0.28a-d	0.43g-k	0.11a-c
26	BOTRY	1.27g-k	0.23a-c	0.33c-j	0.07ab
27	CK801	1.32h-k	0.36a-f	0.30-j	0.14a-e
28	TOG6635	1.22e-k	0.38a-f	0.47i-k	0.13a-d
29	SUPA	1.09b-k	0.28a-c	0.35d-k	0.09ab
30	TOG6241	0.80a-i	0.30a-d	0.55k	0.12a-c
31	CK90	1.41i-k	0.28a-c	0.38f-k	0.12a-b
32	SHAKA102	1.35h-k	0.27a-c	0.48j-k	0.11a-c
	Mean	0.92	0.25	0.26	0.10
	SE	0.36	0.6	0.13	0.05
	CV%	41.9	24.8	74	52.8

Numbers followed by the same letter (s) in columns are not significantly different at $P \leq 0.05$ using Duncan Multiple Range test.

4.2.5 Variation of leaf bronzing among the varieties

The first symptoms of Fe toxicity on the leaf were observed after ten days of Fe treatment (300ppm Fe^{2+}). However, varieties produced different degree of symptoms over the period. Leaf bronzing score after three weeks of Fe stress ranged from 1.8 (ARICA 1) to 6 (Suakok 8) with a mean of 3.6. Suakoko 8 which was used as one of the tolerant check showed a leaf bronzing score higher than the sensitive check IR64 (Table 8).

Table 8: Iron toxicity symptom on rice genotypes after being exposed to 300ppm Fe^{2+} for three weeks

Variety	Leaf bronzing score
ARICA1	1.8
SARO5	2.2
ARICA7	2.3
CK801	2.5
SAHEL	2.5
TXD88	2.5
JASMINE	2.5
ARICA8	2.8
NL23	2.8
ORYLUX4	3.1
ORYLUX6	3.1
SHAKA201	3.1
ARICA6	3.2
IR64	3.3
YUNKENG	3.3
YUNYINE	3.5
CK90	3.5
TOG6635	3.5
TOG16771	3.5
IR841	3.6
ARICA3	3.7
ARICA2	3.8
WITA4	4.0
NL19	4.2
FOFIFA172	4.4
72-5	4.5
BOTRY	4.9
KALAMATA	5.0
SUPA	5.1
TOG6241	5.5
CK73	5.9
SUAKOKO 8	6.0
Mean	3.67
SE	0.99
CV%	54.5

4.2.6 Correlation of leaf bronzing with the other traits

Correlation matrix of the traits revealed positive correlations between traits except with leaf bronzing which was negatively correlated with all the other traits. Plant height was significantly and positively correlated to shoot dry weight and root length while number of leaves was highly significantly correlated with number of tillers. Shoot dry weight was significantly correlated with root length and root weight while root length was significantly correlated to root weight. Leaf bronzing was significantly correlated only with shoot dry weight (Table 9). It means that varieties with high leaf bronzing had small shoot biomass.

Table 9: The correlation matrix Pearson between morphological traits of 32 varieties

	Plant height	**Number of Leaves**	**Number of Tillers**	**Shoot dry wt (g)**	**Root Length (cm)**	**Root dry wt (g)**	**Leaf bronzing**
Plant height	1.000						
Nb of leaves	0.406	1.000					
Nb of Tillers	0.197	0.848**	1.000				
Shoot wt (g)	0.547*	0.449	0.348	1.000			
Root L (cm)	0.600*	0.491	0.346	0.678*	1.000		
Root wt(g)	0.478	0.438	0.394	0.654*	0.577*	1.000	
Leaf bronzing	-0.402	-0.487	-0.407	-0.523*	-0.478	-0.345	1.000

4.3 Genetic Diversity of the Collection Tested and Relationship with Tolerance to Iron Toxicity

4.3.1 Allelic diversity

Out of the 12 markers tested, 10 produced bands of varying molecular weights indicating polymorphism at the respective loci. These 12 SSR markers were used to assess the genetic diversity of 28 rice varieties out of the 32 tested (Table 10). The alleles were recorded at the loci of 12 microsatellite markers across 28 rice varieties. Two to eight alleles per locus were identified with an average of 3.9 alleles per locus. The highest number of alleles (8.0) was found for the locus RM 341and the lowest number of alleles (2.0) was recorded with RM 221, RM 455 and RM 443. A gel image of fragments amplified with RM 341 is shown in Fig. 5 A moderate diversity exists among the 28 rice varieties (0.56 on average) and ranged from 0.21 to 0.83 with an average of 0.56. The major alleles frequency at a given locus ranged from 0.27 (RM 341) to 0.88 (RM 443) as showed in (Table 10).

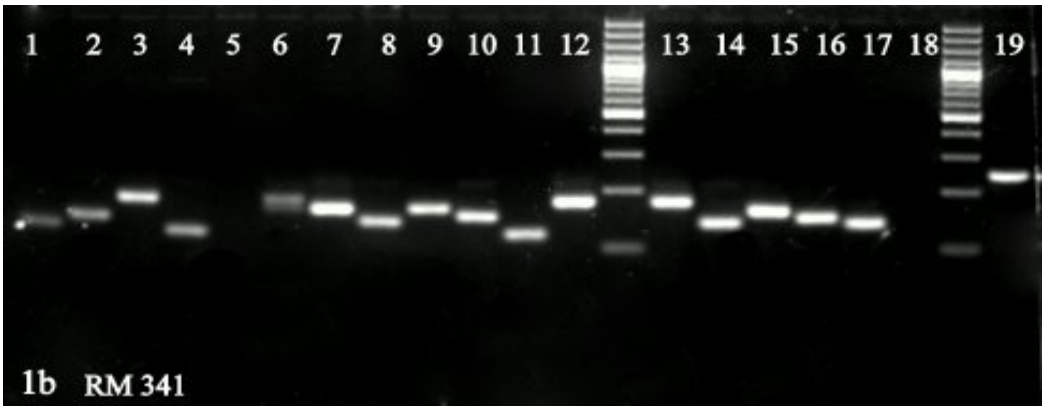

Figure 5: Gel image of PCR amplification products using SSR marker RM 341.

4.3.2 Polymorphism Information Content (PIC)

Polymorphic information content (PIC) values varied from 0.19 to 0.81 with an average of 0.51 (Table 10). The highest PIC value was generated from RM 341 (0.80) followed by RM 6, RM 333 and RM 11529 (0.66).

Table 10: Number of alleles, major allele frequency, genotype number, gene diversity and polymorphism information content (PIC) revealed by 10 SSR markers used to genotype 28 rice varieties

Marker	Major Allele Frequency	Genotype No.	Number of alleles	Availability	Gene Diversity	Heterozygosity	PIC
RM 333	0.33	4.00	4.00	0.86	0.71	0.00	0.66
RM 221	0.76	2.00	2.00	0.75	0.36	0.00	0.30
RM 341	0.27	9.00	8.00	1.00	0.83	0.04	0.81
RM 6	0.34	6.00	5.00	1.00	0.71	0.04	0.66
RM11488	0.64	4.00	4.00	1.00	0.53	0.00	0.48
RM429	0.44	4.00	4.00	0.96	0.63	0.78	0.55
RM 455	0.86	2.00	2.00	1.00	0.24	0.00	0.21
RM443	0.88	2.00	2.00	0.89	0.21	0.00	0.19
RM11522	0.50	5.00	4.00	1.00	0.64	0.04	0.59
RM11529	0.43	5.00	4.00	1.00	0.71	0.04	0.66
Mean	0.55	4.30	3.90	0.95	0.56	0.09	0.51

NB: The loci RM 561 and RM 1154 were not polymorphic and were not included in the analysis.

4.3.3 Pair wise genetic dissimilarity

The pairwise dissimilarity matrix indicated that the lowest genetic dissimilarity (0.08) was between Suakoko 8 and Saro 5 while the highest dissimilarity coefficient (0.88) was between the variety Yunyine and 72-5 (Table 11). The genetic dissimilarity revealed that there were no varieties related at 100%.

Table 11: Pair wise genetic distance indices among 28 varieties obtained from microsatellite marker analysis. Varieties codes (V1 to V31) are as indicated below

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V17	V19	V20	V21	V22	V23	V24	V25	V27	V28	V29	V30	V31
V2	0.56																											
V3	0.25	0.64																										
V4	0.17	0.55	0.20																									
V5	0.17	0.58	0.27	0.27																								
V6	0.17	0.58	0.36	0.27	0.17																							
V7	0.39	0.67	0.36	0.36	0.42	0.33																						
V8	0.22	0.67	0.50	0.36	0.29	0.38	0.38																					
V9	0.50	0.50	0.55	0.45	0.42	0.25	0.33	0.58																				
V10	0.28	0.58	0.18	0.27	0.08	0.25	0.33	0.29	0.42																			
V11	0.61	0.50	0.55	0.45	0.50	0.42	0.33	0.50	0.25	0.50																		
V12	0.39	0.58	0.45	0.36	0.50	0.50	0.25	0.21	0.58	0.42	0.42																	
V13	0.56	0.18	0.70	0.60	0.64	0.64	0.64	0.64	0.64	0.64	0.73	0.55																
V14	0.44	0.55	0.50	0.45	0.50	0.41	0.23	0.59	0.14	0.50	0.23	0.50	0.64															
V15	0.28	0.60	0.33	0.11	0.20	0.10	0.20	0.30	0.30	0.20	0.40	0.40	0.60	0.35														
V17	0.67	0.27	0.50	0.60	0.55	0.64	0.55	0.73	0.64	0.55	0.55	0.55	0.27	0.55	0.60													
V19	0.50	0.73	0.50	0.50	0.45	0.27	0.27	0.41	0.36	0.36	0.45	0.36	0.73	0.50	0.30	0.73												
V20	0.28	0.55	0.30	0.30	0.09	0.18	0.36	0.41	0.36	0.18	0.45	0.45	0.60	0.45	0.30	0.50	0.30											
V21	0.56	0.75	0.64	0.59	0.54	0.46	0.54	0.46	0.54	0.54	0.63	0.54	0.64	0.64	0.45	0.73	0.41	0.59										
V22	0.39	0.73	0.40	0.45	0.27	0.27	0.55	0.50	0.45	0.36	0.55	0.55	0.80	0.65	0.44	0.70	0.20	0.10	0.50									
V23	0.56	0.55	0.70	0.50	0.55	0.45	0.64	0.55	0.41	0.55	0.50	0.50	0.65	0.60	0.56	0.75	0.35	0.45	0.45	0.35								
V24	0.67	0.36	0.80	0.70	0.64	0.73	0.73	0.55	0.73	0.64	0.73	0.55	0.27	0.73	0.70	0.36	0.64	0.70	0.64	0.70	0.55							
V25	0.39	0.42	0.36	0.27	0.42	0.42	0.25	0.46	0.25	0.33	0.25	0.33	0.55	0.23	0.40	0.55	0.45	0.36	0.63	0.55	0.41	0.64						
V27	0.39	0.58	0.36	0.36	0.25	0.33	0.42	0.38	0.42	0.17	0.50	0.50	0.55	0.50	0.30	0.55	0.45	0.36	0.54	0.55	0.55	0.55	0.33					
V28	0.50	0.75	0.36	0.45	0.42	0.42	0.58	0.58	0.50	0.42	0.58	0.58	0.73	0.68	0.50	0.73	0.27	0.27	0.46	0.09	0.41	0.73	0.58	0.58				
V29	0.39	0.50	0.45	0.36	0.25	0.25	0.42	0.42	0.17	0.25	0.33	0.58	0.64	0.23	0.20	0.64	0.45	0.36	0.54	0.55	0.50	0.64	0.33	0.25	0.58			
V30	0.39	0.45	0.40	0.30	0.27	0.18	0.36	0.45	0.18	0.27	0.27	0.55	0.60	0.25	0.20	0.60	0.40	0.36	0.50	0.50	0.35	0.70	0.27	0.27	0.55	0.09		
V31	0.67	0.88	0.73	0.68	0.67	0.75	0.67	0.54	0.75	0.67	0.75	0.63	0.68	0.77	0.70	0.82	0.64	0.64	0.63	0.64	0.64	0.68	0.71	0.54	0.50	0.67	0.73	

CK90=V1, 72-5=V2, SUPA=V3, ARICA1=V4, SARO5=V5, IR841=V6, WITA4=V7, TXD88=V8, SAHEL201=V9, SUAKOKO8=V10, ARICA3=V11, NL19=V12, TOG16771=V13, ARICA6=V14, IR64=V15, TOG6241=V17, KALAMATA=V19, JASMINE85=V20, FOFIFA172=V21, SHAKA102=V22, CK801=V23, TOG6635=V24, ORYLUX 6=V25, CK73=V27, YUNKENG=V28, ORYLUX4=V29, ARICA7=V30 AND YUNYINE=V31.

4.3.4 Genetic groups as revealed by cluster analysis

Phylogenetic analysis using UPGMA revealed the existence of five clusters (Fig. 6). Group 1 included Suakoko 8, Jasmine 85, IR 64, CK 90, IR841, CK 73, Supa, Botry and ARICA1. Orylux 4, ARICA 3, Sahel 201, Orylux 6, ARICA 7, ARICA 3, and ARICA 6 cluster together in group 2. Group 3 contained Wita 4, TXD 88 and NL19. Group 4 comprisedTOG6635, TOG7241, 72-5 and TOG16771. Group 5 contained CK 801, FOFIFA 172, Yunyine, Yunkeng, Shaka 102 and Kalamata.

None of the clusters contained only iron toxicity tolerant varieties or only susceptible varieties. For instance, the tolerant variety ARICA1 clustered together with SUPA, SUAKOKO 8 and CK 73 which were susceptible to iron toxicity in our experimental conditions. However the clusters matched with the species with all *O. glaberrima* varieties in the same cluster (group 4), all *O. sativa japonica* varieties in the same cluster (group 5) and *O. sativa indica* varieties except CK801 and Kalamata in 3 clusters (group 1, 2 and 3).

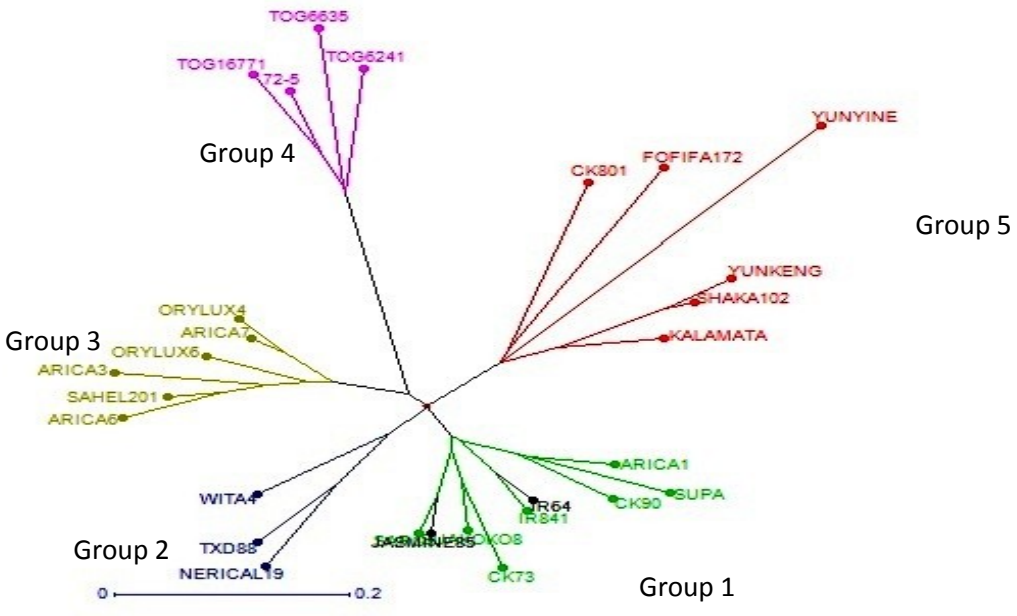

Figure 6: UPGMA Neighbor joining tree of 28 rice genotypes based on allelic data obtained with 10 rice SSR markers

CHAPTER FIVE

5.0 DISCUSSION

Excessive iron uptake has adverse effects on the vegetative growth of rice. It interferes with accumulation of dry matter by destruction of cells in the leaves due to the formation of reactive oxygen species (Becker and Asch, 2005). In this study, the effect of iron Fe^{2+} stress on all parameters was highly significant. Reduction of plant height, leaf and tiller number, shoot and root biomass and root length was observed over the three weeks of Fe stress, confirming that iron toxicity greatly affect rice plants by compromising their growth. This finding is consistent with previous studies which showed that growth traits affected are number of tiller, shoot height, dry weight and decreased dry weight in varieties (Ponnamperuma *et al*., 1955; Olaleye *et al*., 2001; deDorlodot *et al*., 2005). Also similar reductions for all traits were recorded by Priyanga *et al*. (2012). Growth reduction under Fe^{2+} treatment was observed in all the varieties tested but the range was variable. Some varieties showed lower reductions and less symptoms than others. Overall a significant variation was observed between the varieties tested with regards to their relative performances under Fe toxicity stress.

Leaf bronzing score is generally considered as an indicator of Fe toxicity stress level (Bode *et al*., 1995) and it has been often used to select rice varieties tolerant to Fe toxicity. In this study, nine varieties had a leaf bronzing score below 3: ARICA 1, Saro 5, ARICA 7, TXD88, Sahel201, CK801, NL23, ARICA8 and ARICA3 suggesting that they may have some tolerance to Fe toxicity. In terms of biomass accumulation under stress varieties ARICA8, FOFIFA172, TOG6635, CK801, TOG16771, TOG6241, 72-5, NL19, Shaka102, and CK90 had shoot and root biomass above the average. Taken together, CK801 and ARICA 8 which had both low leaf bronzing score and high biomass under

stress could be considered as tolerant to Fe toxicity in our experimental conditions. Surprisingly some of the tolerant checks (Suakoko 8 and WITA4) used in this experiment did not perform well. This is contrary to previous findings which assert that in both field and trials, these varieties combined high yield with iron toxicity tolerance (Gridley *et al*., 2006; Sikirou *et al.,* 2015). During the early stages of the experiment, it was observed that the roots of Suakoko 8 were quickly reaching the bottom of the trays while the other varieties' roots were elongating at a slower rate. It is suspected that the deep root character of SUAKOKO 8 which in the field is beneficial could have been detrimental in hydroponic conditions. Indeed, in field conditions, iron toxicity layer is within the first 2-15cm and varieties that have short root systems are usually susceptible to iron toxicity while traditional varieties with deep root systems tend to be tolerant to Fe toxicity (Benckiser *et al.,* 1983). In hydroponic culture, there is no possibility of escape or avoidance by going deeper.

The variation of morphological traits between tolerant rice varieties and susceptible ones suggest the presence of different alleles linked to these traits. Cluster analysis of the allelic data generated in this study divided the varieties into five clusters. However, the results indicated that grouping seemed to be based on species and genetic background rather than on iron toxicity tolerance level. In fact tolerant varieties did not group together and in some clusters both tolerant and susceptible varieties were present. Also using SSR markers, Onaga *et al*. (2013), found that there was a low correlation between molecular and morphological variation. This means that molecular markers used in both studies cannot be used to infer varieties tolerance to iron toxicity. On the other hand, the SSR markers used in this study provided adequate power of resolution to discriminate between varieties as indicated by the genetic distance and cluster analysis. Among all the markers tested RM341 was the most polymorphic one. It was also found by Onaga *et al*. (2013) to

be one of the markers that best discriminated the varieties tested in that study. Such an informative marker should be included in future genetic studies. Genetic diversity revealed with relatively few markers in this study confirms the power of SSR markers for genetic diversity analysis in rice as reported earlier in various studies (Onaga *et al*., 2013; Jeung *et al.,* 2005; Xu *et al.,* 2004). Using molecular markers such as the SSR described here, to assess genetic relatedness of crossing parents, is valuable information for breeding programs. The use of genetically distant parents increases the variation within the population and widens the gene pool of the breeding program.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

A good genetic variation for Fe toxicity tolerance was found among the varieties tested. The varieties CK801 and ARICA8 are tolerant to iron toxicity as proven by their better performances under Fe toxicity stress. These varieties can be used in breeding programs to increase rice production in iron toxic areas. Molecular markers used in this study were able to distinguish the different rice species but could not clearly distinguish Fe toxicity tolerant from susceptible varieties.

6.2 Recommendations

i. Tolerances of selected varieties need to be confirmed in field conditions.

ii. The SSR markers in this study are highly informative markers and are recommended for a rapid assessment of the genetic diversity among genotypes.

iii. An initiative to improve farmers' preferred varieties should be taken since iron toxicity tolerant parents are available.

Development of new markers tightly associated with Fe toxicity tolerance genes (SNPs) rather than flanking markers is needed in future genetic analysis of Fe toxicity.

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APPENDICES

Appendix 1: Random assignment of two Fe concentrations (2ppm and 300ppm Fe^{2+}) in main plots and 32 rice varieties in sub plots in a split plot design replicated four times

Rep 1 Fe(-)	V30	V17	V12	V3	V24	V18	V32	V5
	V16	V11	V14	V1	V26	V23	V20	V15
	V28	V7	V25	V4	V2	V10	V22	V9
	V31	V21	V13	V19	V6	V8	V27	V29

Rep 2 Fe(+)	V9	V12	V16	V19	V28	V20	V14	V32
	V5	V21	V17	V2	V25	V18	V3	V15
	V10	V22	V7	V31	V23	V30	V8	V26
	V29	V11	V13	V4	V27	V24	V21	V6

Rep3 Fe(-)	V15	V21	V19	V32	V14	V28	V5	V10
	V22	V27	V20	V31	V4	V7	V17	V6
	V18	V23	V24	V30	V12	V1	V13	V11
	V29	V2	V16	V3	V9	V25	V8	V26

Rep4 Fe(+)	V13	V15	V24	V11	V21	V26	V29	V14
	V19	V32	V27	V8	V22	v23	V2	V12
	V17	V10	V9	V5	V1	V25	V4	V30
	V6	V28	V18	V20	V3	V16	V7	V31

Rep1 Fe(+)	V4	V13	V5	V24	V3	V7	V20	V2
	V23	V26	V11	V22	V9	10	V28	V16
	V12	V29	V30	V25	V31	V15	V14	V18
	V6	V32	V8	V1	V21	V17	V19	V27

Rep2 Fe(-)	V27	V16	V13	V9	V7	V23	V29	V25
	V8	V18	V1	V17	V24	V19	V26	V10
	V32	V5	V31	V22	V15	V20	V21	V3
	V2	V11	V28	V4	V12	V6	V30	V14

Rep3 Fe(+)	V19	V18	V5	V13	V23	V24	V4	V20
	V16	V26	V14	V9	V32	V29	V10	V2
	V1	V17	V21	V8	V31	V28	V15	V11
	V12	V3	V7	V22	V30	V6	V27	V25

Rep4 Fe(-)	V2	V24	V18	V16	V12	V9	V6	V25
	V17	V1	V30	V4	V14	V28	V5	V11
	V20	V19	V26	V29	V3	V23	V13	V32
	V22	V31	V10	V15	V27	V21	V8	V7

Appendix 2: Research pictorial both at screanhouse and laboratory