

**DIVERSITY FOR ZINC AND IRON CONTENT IN BLACK
RICE (*Oryza sativa* L.)**

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**DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BENGALURU-560065**

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RICE (*Oryza sativa* L.)**

**KRISHNAMURTHY, K.V
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*Affectionately dedicated to
my beloved Parents, Guide,
Brother and Friends.*

**UNIVERSITY OF AGRICULTURAL SCIENCES
DEPARTMENT OF PLANT BIOTECHNOLOGY
BENGALURU-560065, INDIA**

CERTIFICATE

This is to certify that the thesis entitled “**Diversity for Zinc and Iron content in Black rice (*Oryza sativa* L.)**” submitted by **Krishnamurthy K.V., ID. No. PALB-2247** in partial fulfilment of the requirement for the degree of **Master of Sciences (Agriculture)** in **Plant Biotechnology** to the University of Agricultural Sciences, Bengaluru, is a record of bonafide research work done by him during the period of his study in this University under my guidance and supervision and that no part of the thesis has previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar titles.

Bengaluru

July, 2015

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“Gratitude is the memory of the heart”

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Diversity for Zinc and Iron Content in Black Rice **(*Oryza sativa* L.)**

Krishnamurthy K.V

ABSTRACT

Rice is the most important staple food crop of world, which provides 20% of world's dietary energy requirement. Among the rice types, black rice was reported to have high nutritive value and is the main source of vitamin-E. In the present study eight rice cultivars were used to evaluate zinc and iron content, and for molecular characterization based on candidate genes.

The experiment was laid out in RCBD design during 2012-13, at Department of Plant Biotechnology, UAS, GKVK, Bengaluru. Various plant traits, in addition to zinc and iron content were evaluated. Significant differences were observed for most of the plant traits viz., plant height, grain yield/plant and straw yield/plant, root number, root volume. While, for zinc and iron content significant differences were observed in all parts of the plant studied. These results indicated considerable amount of variation existed in rice cultivars used.

Higher GCV, PCV coupled broad sense heritability and GAM was observed for plant height, grain yield per plant, root number and volume. For zinc and iron content, higher values were observed for zinc in culm and zinc and iron in root. Revealing these traits are under genetic control and responds directly for selection. Correlation study revealed significant negative correlation between straw yield/plant and grain iron content.

Molecular characterization was carried out using 29 candidate gene specific primers for zinc and iron transporters. All primers showed monomorphic banding pattern. Further, sequencing of these fragments may reveal SNPs for functional association.

June, 2015

UAS, GKVK, Bengaluru – 65

Signature of major advisor

(H. E. Shashidhar)

ಕಪ್ಪು ಭತ್ತದಲ್ಲಿನ ಸತು ಹಾಗೂ ಕಬ್ಬಿಣ ಅಂಶದ ವೈವಿಧ್ಯತೆ

ಕೃಷ್ಣಮೂರ್ತಿ, ಕೆ. ವಿ

ಪ್ರಬಂಧದ ಸಾರಾಂಶ

ಭತ್ತವು ವಿಶ್ವದ ಪ್ರಮುಖ ಏಕದಳ ಆಹಾರ ಬೆಳೆ ಮತ್ತು ಪ್ರತಿಶತ ೨೦ ರಷ್ಟು ಆಹಾರ ಶಕ್ತಿಯ ಮೂಲವಾಗಿದೆ. ಭತ್ತದ ವಿಧಗಳಲ್ಲಿ ಕಪ್ಪು ಭತ್ತವು ಹೆಚ್ಚು ಪೋಷಿಕಾಶಗಳು ಮತ್ತು ವಿಟಮಿನ್ ಇ ಹೊಂದಿದೆ ಎಂದು ಅಧ್ಯಯನದ ವರದಿಗಳಿಂದ ತಿಳಿದು ಬಂದಿದೆ. ಈ ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ ಕಪ್ಪು ಭತ್ತ ಸೇರಿದಂತೆ ಎಂಟು ತಳಿಗಳ ಆಯ್ಕೆ ಮಾಡಿ ಆರ್ ಸಿ ಬಿ ಡಿ ವಿನ್ಯಾಸ ಬಳಸಿಕೊಂಡು ಯುಎಎಸ್, ಜಿಕೆವಿಕೆ ಬೆಂಗಳೂರಿನ ಸಸ್ಯ ಜೈವಿಕ ತಂತ್ರಜ್ಞಾನ ವಿಭಾಗದಲ್ಲಿ ಸಸ್ಯದ ವಿವಿಧ ಲಕ್ಷಣಗಳ ಜೊತೆಗೆ ಸಸ್ಯದ ಎಲ್ಲಾ ಭಾಗಗಳ ಸತು ಹಾಗೂ ಕಬ್ಬಿಣದ ಅಂಶವನ್ನು ಅಭ್ಯರ್ಥಿ ಜೀನ್‌ಗಳನ್ನು ಆಧರಿಸಿ ಅಣ್ವಿಕ ಅಣು ಪರಿಶೀಲನೆ ಮಾಡಲಾಯಿತು. ಸಸ್ಯದ ಎತ್ತರ, ಧಾನ್ಯ ಇಳುವರಿ, ಮೂಲಪರಿಮಾಣ, ಹುಲ್ಲು ಇಳುವರಿ, ಮೂಲಸಂಖ್ಯೆ ಹಾಗೂ ಸತು ಮತ್ತು ಕಬ್ಬಿಣದ ಅಂಶವು ಸಸ್ಯದ ಎಲ್ಲ ಭಾಗಗಳಲ್ಲಿ ಗಮನಾರ್ಹ ವ್ಯತ್ಯಾಸಗಳು ಕಂಡುಬಂದವು ಈ ಫಲಿತಾಂಶಗಳು ಗಣನೀಯ ಪ್ರಮಾಣದ ಬದಲಾವಣೆಗಳನ್ನು ಭತ್ತದ ತಳಿಗಳಲ್ಲಿ ಸೂಚಿಸಿದವು, ಹೆಚ್ಚಿನ ಜಿ.ಸಿ.ವಿ, ಪಿ.ಸಿ.ವಿ ಮತ್ತು ಜಿ.ಎ.ಎಮ್ ಮೌಲ್ಯಗಳನ್ನು ಸಸ್ಯ ಎತ್ತರ, ಧಾನ್ಯ ಇಳುವರಿ ಬೇರುಗಳ ಸಂಖ್ಯೆ, ಬೇರುಗಳ ಪರಿಮಾಣ ದಾಖಲಾದವು. ಹೆಚ್ಚಿನ ಸತುವಿನ ಅಂಶವು ಕಲ್ಲಿದಲ ಧೂಳುಗಳಲ್ಲಿ ಮತ್ತು ಬೇರುಗಳಲ್ಲಿ ಹಾಗೂ ಕಬ್ಬಿಣದ ಅಂಶವು ಬೇರುಗಳಲ್ಲಿ ದಾಖಲಾಯಿತು.

ಈ ಲಕ್ಷಣಗಳು ಅನುವಂಶೀಯ ನಿಯಂತ್ರದಲ್ಲಿರುತ್ತವೆ ಮತ್ತು ಆಯ್ಕೆಗೆ ನೇರವಾಗಿ ಪ್ರತಿಕ್ರಿಯಿಸುತ್ತವೆ, ಸಸ್ಯ ಹುಲ್ಲು ಇಳುವರಿಯು ಗಮನಾರ್ಹ ಋಣಾತ್ಮಕ ಸಂಬಂಧವನ್ನು ಧಾನ್ಯ ಕಬ್ಬಿಣದ ಅಂಶದೊಂದಿಗೆ ಹೊಂದಿದೆ ಎಂದು ತಿಳಿದು ಬಂದಿತು. ಕಬ್ಬಿಣ ಮತ್ತು ಸತುವನ್ನು ನಿರ್ದಿಷ್ಟವಾಗಿ ಸಾಗಣೆ ಮಾಡುವ ೨೯ ಅಭ್ಯರ್ಥಿ ಜೀನ್ ಗುರುತುಕಾರಗಳನ್ನು ಬಳಸಿ ಅಣ್ವಿಕ ವಿಶ್ಲೇಷಣೆ ಮಾಡಲಾಯಿತು. ಎಲ್ಲಾ ಗುರುತುಕಾರಗಳು ಏಕರೂಪದೇಯ ಬ್ಯಾಡಿಂಗ್ ಮಾದರಿಯನ್ನು ಪ್ರದರ್ಶಿಸಿದವು. ಮತ್ತಷ್ಟು ಈ ಬ್ಯಾಂಡ್‌ಗಳು ಅನುಕ್ರಮ ಸಿಕ್ವೆನ್ಸಿಂಗ್ ಮಾಡುವುದರಿಂದ ಎಸ್.ಎನ್.ಪಿ ನ ಕ್ರಿಯಾತ್ಮಕ ಜೊತೆಗಾರಿಕೆಯನ್ನು ಬಹಿರಂಗಪಡಿಸಬಹುದು.

ಜೂನ್, ೨೦೧೫

ಕೃ.ವಿ.ವಿ. ಬೆಂಗಳೂರು-೫೬೦ ೦೬೫

ಪ್ರಮುಖ ಸಲಹೆಗಾರರು

(ಹೆಚ್, ಇ. ಶಶಿಧರ್)



“Molecular Analysis of Zinc and Iron in Black Rice”

Krishnamurthy K.V. and H.E Shashidhar

Department of Plant Biotechnology, University of Agriculture Sciences, GKVK, Bangalore-560065.



Introduction

- Rice is central to the lives of billions of people around the world being the staple food for 2.5 billion people
- Micronutrients deficiency is a global problem contributing to world's widespread malnutrition for high rate of children and women mortality
- Zinc deficiency is positioned at fifth rank among the most important health risk factors in developing countries
- Increasing micronutrient content like Iron and Zinc content in grains of rice will bring down the health problems induced by its deficiency
- Biofortification is important strategy to improve the micronutrient content in the rice grains

Objectives

- Quantification of iron and zinc in grains, root, culm, leaves of black rice
- Molecular analysis of candidate gene markers for zinc transporters

Materials and methods

- The study consists diversified rice accessions (BI33, Azucena, IR20, Jeerigesanna, Black Rice, Devamallige, AM 65 and BJ21)
- The seeds were sown in the pipe with diameter of 15cm and height of 60 cm in three replications and post harvest study of root morphology parameters in pipe experiment conducted
- All biometrical parameters were recorded
- Iron and Zinc content was estimated using AAS (Atomic Absorption Spectrophotometer)
- Candidate gene markers were used for molecular analysis of zinc and iron

Recording of observations:

- Plant height
- Panicle length
- Number of tillers
- Number of productive tillers
- Grain yield/Plant
- Straw yield/Plant
- Root fresh weight
- Root length
- Root numbers
- Root volume
- Days to 50% flowering

Results

Character	Min	Max	Mean
Plant height (cm)	51.00	118.00	96.62
Panicle length (cm)	15.00	27.00	20.28
Nor of tillers	09.00	15.00	12
No of productive tillers	08.00	13.00	10.00
Grain yield / plant	03.84	19.46	11.70
Straw yield / plant	10.33	27.33	20.09
Root fresh weight	28.67	88.67	55.85
Root length	46.00	80.00	63.48
Root numbers	57.05	106.00	75.00
Root volume	40.00	71.02	46.00
Days to 50% flowering	96.00	118.00	103.00

Phenotypic parameters for different quantitative yield traits

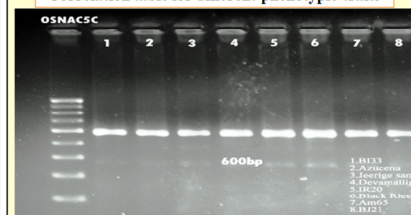
	Zn Grain	Zn Leaf	Zn Culm	Zn Root	Fe Grain	Fe Leaf	Fe Culm	Fe Root
Zn Grain	1	-.651*	.201	-.406	-.119	.007	-.019	-.036
Zn Leaf		1	-.358	.327	-.329	-.537	-.154	-.347
Zn Culm			1	-.189	.821**	-.333	.279	.032
Zn Root				1	-.032	-.103	.257	.509
Fe Grain					1	-.044	.503	.127
Fe Leaf						1	.242	.293
Fe Culm							1	-.148
Fe Root								1

*. Correlation is significant at the 0.05 level. **. Correlation is significant at the 0.01 level

Correlation table for zinc and iron content of grain, leaf, culm and root

Character	Plant height	Panicle length	Number of tillers	Number of productive tillers	Yield(g) / plant	Straw yield(g) / plant	Root length	Root number	Root volume	Days to 50% flowering
Plant height	1	0.68	-0.308	-0.22	-0.396	0.191	0.418	0.651	-0.046	0.26
Panicle length		1	-0.654	-0.502	-0.01	-0.19	0.117	0.382	0.05	-0.117
Number of tillers			1	0.421	-0.324	0.383	-0.292	-0.281	-0.658	-0.258
Number of productive tillers				1	0.332	0.404	-0.11	-0.087	-0.405	-0.408
Yield(g) / plant					1	0.074	-0.505	-0.018	0.09	-0.281
Straw yield / plant						1	0.173	0.658	0.089	0.362
Root fresh weight							1	0.488	0.598	0.893**
Root length								1	0.311	0.549
Root number									1	0.744*
Root volume										0.372
Days to 50% flowering										1

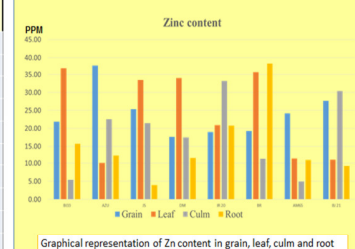
Correlation table for different phenotypic traits



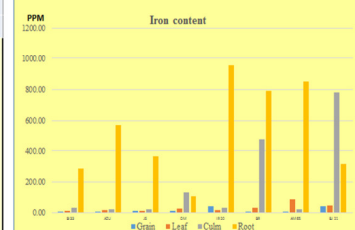
Agarose gel picture of OsNAC

Discussion

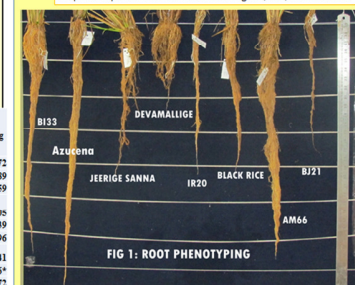
- Grain Zn content was significantly correlated with grain Fe and culm Fe content
- Among phenotypic traits maximum root length observed was 80cm and root number was 106 numbers improving these traits will help in breeding for drought tolerant varieties
- Correlation studies revealed that root fresh weight was significantly correlated with root volume, this correlation studies also suggesting that improving root traits will also help in development of drought tolerant varieties



Graphical representation of Zn content in grain, leaf, culm and root



Graphical representation of Fe content in grain, leaf, culm and root



Roots after sampling

Summary

- Zinc and Iron accumulation in grains, leaf, culm and roots was estimated in the diverse rice accessions. High grain Zn content was observed in Azucena but high Zn content in leaf, culm and roots found more in BI33, IR20 and Black rice respectively.
- High Iron content was found in roots of all genotypes except BJ21

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I. INTRODUCTION

Rice (*Oryza sativa* L.) is the predominant staple food for 17 countries in Asia and the Pacific, nine countries in North and South America and eight countries in Africa. Rice provides 20 percent of the world's dietary energy supply, while wheat supplies 19 percent and maize 5 percent, rice is a good source of thiamine, riboflavin and niacin. Unmilled or Brown rice contains a significant amount of dietary fibre. The amino acid profile of rice shows that it is high in glutamic and aspartic acid, while lysine is the limiting amino acid (www.rice2004.org).

Black rice used to be known as the “forbidden rice.” Ancient emperors in China hoarded black rice for the reason that they believe it would lengthen their lives and secure their thrones longer (www.behealthyandwell.com).

They may have been right, as research now shows that black rice is one of the world's “super foods.” The purplish-blackish pigments are also antioxidants which are known as anthocyanin the same ones which are in blueberries said to be one of the richest sources of antioxidants, which is known for high fiber, minerals and vitamins than the regular rice varieties (www.chinese-black-rice.com).

Black rice, has been shown to possess bioactive properties, and rice bran contains high levels of several antioxidant compounds. Relatively high levels of free polyphenols and flavonoids found in bran and lower levels were found in endosperm (Kong and Lee, 2010).

It is estimated that the per capita consumption of rice ranges from 62 to 190 kg/year in countries where it is used as staple food (Graham *et al.*, 1999). Rice provides approximately 20 percent of the per capita energy and 13 percent of the protein for human consumption worldwide. In many developing countries the dietary contribution of rice is 29.3 and 29.1 percent for energy and protein, respectively (Sautter *et al.*, 2006).

The poorest families in developing countries spend 50 – 80 percent of their income on food, depend heavily on low cost, high energy, starchy staples like rice which contains insufficient amounts of micronutrients such as Zn, Fe and vitamin A to provide recommended daily intake for human beings (Goto *et al.*, 1999; Gregorio *et al.*, 1999). Southeast Asia is the major rice producer in the world where 71 percent of population suffers from zinc deficiency.

It has been estimated that about 50 per cent world's population depends on rice as their main source of nutrition (White, 1994). Micronutrients deficiency is a global problem contributing to world's widespread malnutrition resulting in high rate of children and women mortality (WHO, 1996). Zinc deficiency is positioned at fifth rank among the most important health risk factors in developing countries and 11th position worldwide (WHO 2002). Zn deficiency causes a lot of health imbalances in humans, such as impairments of physical development, immune system, and brain function (Cakmak,

2008). According to WHO (2011) approximately 1.62 billion people suffer from iron deficiency (anemia) and more than half of world population suffers from Zn deficiency.

In plants functions like formation of starch, chlorophyll and proper root development. Zn deficiency will lead to Khaira disease in plants and as a result yield will reduce. Most of zinc in the rice grains is located on the outer layers (bran) but its appreciable amount is reduced by polishing. The concentration of zinc in rice bran (aleurone and embryo tissue) is 50.3ppm compared to 8.6ppm in the endosperm. Polished rice grains contain only about 20 percent of the daily requirement of zinc (Virk *et al.*, 2007).

Increasing micronutrient content like iron and zinc content in grains of rice will bring down the health problems induced by its deficiency (Graham *et al.*, 1999; WHO, 2002; Martinez *et al.*, 2007).

Applying chemical fertilizer to crops to enhance Zn content is expensive and pollutes the environment. Fortification of zinc and supplementation approaches also can be selected but these kinds of methods are expensive and it is not easily accessible for people in developing countries. (Bouis, 2003; Stein *et al.*, 2007; Pfeiffer and Mc Clafferty, 2007). Biofortification of the zinc content using conventional breeding and biotechnological methods can enhance the nutrient content in grains of rice. This method is cheaper, stable and can reach the local poor society (Bouis, 2003; Pfeiffer, Mc Clafferty, 2007). The genetic basis of accumulation of micronutrients in the grains and mapping of the quantitative trait loci (QTL) will provide the basis for preparing the strategies and improving grain micronutrient content through marker assisted selection.

Efforts have been made for the improvement of Zn content in rice grain for greater quantities of Zn by selection of mineral efficient varieties with high yield and accumulate minerals from infertile soils, thus enhanced bioavailable Zn in edible portions through increasing the concentrations of metal binding proteins (Goto, 1999; Gregorio 1999). Realizing the importance of biofortification, several studies were undertaken for the evaluation of germplasm and advance breeding lines for grain Zn content (Gregorio *et al.*, 1999; Lu *et al.*, 2008) Selection of varieties with traits for higher Zn content using marker assisted selection in rice grain is an effective strategy to address widespread dietary deficiency in human populations.

Micronutrient malnutrition normally occurs when the essential elements are not to fulfill the need of the body to develop and function normally. Iron, Zinc are the most occurring nutritional deficiencies in the world (WHO, 2004).

Iron (Fe^{2+}) is an essential nutrient for plants as it is involved in many biological processes, such as photosynthesis, respiration, nitrogen assimilation chlorophyll biosynthesis and as a component of Fe-cluster containing enzymes (Marschner, 1995). Iron deficiency is the most widespread micronutrient deficiency in humans. Iron deficiency in rice can induce chlorosis and decrease photosynthetic activity, leading to lower plant productivity (Hansen *et al.*, 2006). It participates in electron transfer through

reversible redox reactions, cycling between Fe^{2+} and Fe^{3+} insufficient Fe uptake leads to Fe deficiency symptoms such as interveinal chlorosis in leaves and reduction of crop yields (Guerinot and Yi, 1994).

Earlier reports have shown that grain zinc content in rice is governed by a number of QTLs located on different regions of the chromosome with different phenotypic effects (Avendano, 2000; Biradar *et al.*, 2007; Lu *et al.*, 2008; Garcia-Olivera *et al.*, 2009; Zhang *et al.*, 2011). To use this approach preliminary steps are required to characterize molecular markers linked to QTLs for grain Zn content and study their phenotypic variation.

Biofortification is one of the best methods to alleviate malnutrition and development of new cultivars with elevated concentrations of micronutrients using conventional breeding and biotechnological approaches (Graham *et al.*, 1999; Zimmermann and Hurrell, 2002). The main advantages of biofortification compared to other strategies are (i) cost effectiveness (ii) sustainability (iii) it can reach relatively remote poor rural people dependent on rice as staple diet (Bouis, 2003; Pfeiffer and McClafferty, 2007).

Over many years, molecular marker technology has advanced well and this technology will help in genetic mapping and breeding process efficient and faster. DNA markers which are closely linked with desired traits allow the selection of plants possessing those traits prior to trait expression.

The present research was carried out using eight genotypes, with the following objectives

Objectives:

- 1) Phenotypic characterization of zinc and iron content in grains, leaves, culm and roots of black rice and selected rice accessions under aerobic condition.
- 2) Molecular analysis of candidate gene markers associated with zinc and iron content in rice.

II. REVIEW OF LITERATURE

In this chapter available literature pertaining to the present investigation has been reviewed and presented under the following headings.

1. Importance of zinc and iron in humans and plants
2. Aerobic rice
3. Biofortification
4. Variability, Heritability, Genetic advance
5. DNA Markers and their linkage to grain zinc and iron content

1. Importance of zinc and iron in humans and plants

1.1 Zinc

Zinc is one of the most ubiquitous micronutrient essential for plants, animals and humans. The human body requires at least 49 nutrients that play an essential role in a vital role as structural and functional components for a large number of proteins/enzymes. The cysteine, histidine and glutamate or aspartate residues represent the most critical Zn binding sites in enzymes (Welch, 2001, Welch and Graham, 2004).

Major sources of zinc are red meat, poultry, fish and seafood, whole grains, and dairy products which is not affordable for people living in developing countries. The bioavailability of zinc in plant-based foods is generally lower due to dietary fiber and phytic acid which inhibit the absorption of zinc (http://www.iza.com/zinc_health.html).

It is reported that zinc toxicity in crops is far less widespread than Zn deficiency. However, Zn toxicity occurs in soils contaminated by mining and smelting activities, in agricultural soils treated with sewage sludge, and in urban and peri-urban soils enriched by anthropogenic inputs of Zn, especially in low pH soils (Chaney, 1993).

Toxicity symptoms usually become visible at [Zn] leaf ≥ 300 mg Zn kg⁻¹ leaf DW, although some crops show toxicity symptoms at [Zn] leaf ≤ 100 mg Zn kg⁻¹ DW (Chaney, 1993; Marschner, 1995).

Gregorio *et al.*, (1999) found that high Zn content in grains is not only used for human nutrition but also serve as more vigorous and better able to withstand weed competition, denser stands, higher stress tolerance to pathogen and pest attack.

According to WHO (2002) zinc deficiency ranks fifth among the most important health risk factors in developing countries and eleventh worldwide. It is estimated that more than half of world's population suffers from Zn deficiency and majority of them are

from developing countries that are highly dependent on cereal crops such as rice for their sustenance.

WHO (2002) estimated 800,000 deaths worldwide each year to zinc deficiency and over 28 million healthy life years lost. Almost half of these healthy life years are lost in Africa and another 34% in South East Asia. In the developed world, more than 130,000 healthy life years are lost because of zinc deficiency.

Zinc deficiency in human body causes undesirable consequences including growth retardation, dermatitis, impaired immune functioning, hypogonadism, delayed wound healing and poor mental development (WHO, 2002). Thus, intake of a few extra milligrams of zinc each day can make the difference between illness and a healthy life for millions of people around the world, and productive life.

Zinc requirement is increased during pregnancy as well as throughout childhood and adolescence. The daily recommended dietary allowance is 3 - 5, 10, 12, 15 and 16 - 19 mg/day for infants, children, adult women, adult men and breast feeding women, respectively (Dietary Allowances for Americans, 2005).

Zinc is involved in many important cellular functions, protein metabolism, gene expression, structural and functional integrity of cellular membranes, chlorophyll biosynthesis, photosynthesis, respiration and seed maturation (Nishizawa, 2005). It is a cofactor for a number of enzymes including alcohol dehydrogenase, carbonic anhydrase, Cu/Zn-superoxide dismutase, alkaline phosphatase, phospholipase, carboxypeptidase and RNA polymerase.

The most common reason for high Zn deficiency in humans is inadequate dietary zinc intake, particularly in the regions where soils are low in zinc and cereal based foods are the major source of calories (Virk *et al.*, 2007).

Micronutrient deficiency is a global health problem contributing to high rate of children and women's mortality. It is estimated that more than 3.5 billion people in the world are deficient in vitamin A, iodine, iron or zinc (WHO, 1996, Virk *et al.*, 2007).

Zinc deficiency in plants cause decrease in membrane integrity, susceptibility to heat stress, decreased synthesis of carbohydrates, cytochromes, nucleotides, auxin and chlorophyll (Marschner, 1995). Typical symptoms for Zn deficiency in plants include high plant mortality, stuntedness, chlorosis and necrosis on the leaves and a delay in flowering (Broadley *et al.*, 2007).

In plants functions like formation of starch, chlorophyll and proper root development. Zn deficiency will lead to Khaira disease in plants and as a result yield will reduce. Most of zinc in the rice grains is located on the outer layers (bran) but its appreciable amount is reduced by polishing. The concentration of zinc in rice bran (aleurone and embryo tissue) is 50.3ppm compared to 8.6ppm in the endosperm. Polished

rice grains contain only about 20 percent of the daily requirement of zinc (Virk *et al.*, 2007).

Ravindrababu *et al.*, (2011) reported zinc values among tested 173 varieties, Zn concentrations (mg kg⁻¹) varied from 10.1 (Karjat 3) to 32.7 (Ratna).

Studies by HarvestPlus showed that there is a significant G × E interaction in the expression of iron and zinc in the grain. Experiments carried out in Colombia, the Dominican Republic, and Nicaragua reported similar results. In grain Zn content, G × E and G × soil Zn interactions showed significance (Pandian *et al.*, 2011).

Anuradha *et al.*, (2012) reported zinc values of range between 26.2 ppm to 67.3 ppm among tested 126 brown rice varieties.

Moderate PCV and GCV observed for days to 50% flowering, plant height and grain zinc content. These values indicated considerable amount of variability for these characters. Grain zinc content by Ravindrababu *et al.*, (2012).

Ravindrababu *et al.*, (2013) reported mean micronutrient values of 15 hybrids as 37.3 ppm. Two hybrids with both high iron and zinc concentration (DRRH-2 with 125.8 ppm and 43.8 ppm; and Sahyadri-4 with 104.8 and 43.0 ppm) were identified.

1.2 Iron

Iron is an essential micronutrient in plants & animals. Iron is a transition metal essential for virtually all living organisms. Because of its singular capacity to gain or lose electrons, it has crucial participation metabolic processes such as oxido-reductive reactions of biological process. Iron is very abundant in the soil, but is normally chelated to organic matter in oxidized and insoluble forms.

Iron deficiency is a common disorder affecting plants in many areas of the world, and is chiefly associated with high pH and calcareous soils. The low solubility of Fe compounds in many soils prevents plant Fe uptake and indicates the development of Fe deficiency symptoms.

Recent understanding of plant metabolism has made it possible to increase the iron, zinc and β-carotene (provitamin A) content in staple foods by both conventional plant breeding and genetic engineering. Improving the micronutrient composition of plant foods may become a sustainable strategy to combat deficiencies in human populations, replacing or complementing other strategies such as food fortification or nutrient supplementation (Zimmermann *et al.*, 2002).

Sperotto *et al.* (2010) analyzed the expression of 25 metal-related genes from rice, including rice homologues for *YSLs*, *NRAMPs*, *ZIPs*, *IRT1*, *VIT1* (coding for known or potential metal transporters), as well as *NASs*, *FROs* and *NAC5* (involved in metal homeostasis) in flag leaves of eight rice cultivars (showing contrasting levels of seed Fe

and Zn) during panicle emergence (R3) and grain filling stage (R5). The expression level of nine of these genes (*OsYSL6*, *OsYSL8*, *OsYSL14*, *OsNRAMP1*, *OsNRAMP7*, *OsNRAMP8*, *OsNAS1*, *OsFRO1* and *OsNAC5*) in flag leaves exhibited significant correlations with Fe and/or Zn concentrations in the seeds. In this way, they provided a short list of putative target genes to manipulate Fe and Zn concentrations in rice grains.

1.2.1 Iron bioavailability and biogenesis in plants:

Iron is an important nutrient for plant growth, yet the insoluble state of iron hydroxides in alkaline conditions decreases its bioavailability. Iron chelates increase iron uptake and have been used in agriculture to correct iron chlorosis

Lucca *et al.* (2001) introduced a ferritin gene from *Phaseolus vulgaris* into rice grains, increasing their iron content up to two-fold. To increase iron bioavailability, they introduced a thermotolerant phytase from *Aspergillus fumigatus* into the rice endosperm. In addition, as cysteine peptides are considered a major enhancer of iron absorption, over expressed the endogenous cysteine-rich metallothionein-like protein. The content of cysteine residues increased about seven-fold and the phytase level in the grains about 130-fold, giving a phytase activity sufficient to completely degrade phytic acid in a simulated digestion experiment. High phytase rice, with increased iron content and rich in cysteine peptide, has the potential to greatly improve iron nutrition in rice-eating populations.

Hasegawa *et al.* (2011) reported that effects of chelating ligands on iron solubility and bioavailability, they elucidates the pattern of iron movement by chelating ligands in plant growth medium. Ethylene diamine disuccinic acid (EDDS) and hydroxy-imino disuccinic acid (HIDS) produced the highest apparent mobility of iron from the bottom layer of the medium (initially 10–4M Fe(III) to the upper layer (no iron), followed by glutamate diacetic acid (GLDA), ethylene diamine tetraacetic acid (EDTA), methyl glycine diacetic acid (MGDA), and iminodisuccinic acid (IDS). Iron movement in the growth medium was influenced by the chelating ligand species, pH, and ligand exposure time. The iron uptake and growth of radish sprouts were related to the iron mobility produced by the chelating ligands. These results suggest that, in alkaline media, chelating ligands dissolve the hardly soluble iron hydroxide species, thus increasing iron mobility, iron uptake, and plant growth.

1.2.2 Iron uptake

Mainly they are two major strategies for acquisition in higher plants (Romheld, 1987).

Strategy 1: (non-graminaceous monocots & dicots)

- ▶ Reduction based strategy
- ▶ By proton release or Fe(III)-chelate reduction

- ▶ More soluble Fe^{2+} is then transported to roots

Strategy 2: (graminaceous monocots)

- ▶ Release mugenic acid family phytosiderophores, binds Fe^{3+} in rhizosphere
- ▶ Fe^{3+} -PS complex transported to plant roots

Graminaceous plants secrete mugineic acids (MAs) from their roots to solubilize rhizospheric Fe (III) (Takagi, 1976). Subsequently, Fe (III)–MAs complexes are taken up through the Fe (III)–MAs transporter, *YS1* (Curie *et al.*, 2001). In rice and barley, the *YS1* homologs *OsYSL15* and *HvYSL1* transport Fe (III)–MAs complexes (Inoue *et al.*, 2003; Murata *et al.*, 2006). MAs are biosynthesized from methionine through nicotianamine (Shojima *et al.*, 1989). NA synthase (NAS) synthesizes NA from three molecules of S-adenosyl-methionine (Higuchi *et al.*, 1999, 2001; Douchkov *et al.*, 2002; Inoue *et al.*, 2003; Mizuno *et al.*, 2003).

In response to iron deficiency, Arabidopsis roots induce the expression of the divalent cation transporter *IRT1*. Here, they present genetic evidence that *IRT1* is essential for the uptake of iron from the soil. An Arabidopsis knockout mutant in *IRT1* is chlorotic and has a severe growth defect in soil, leading to death. This defect is rescued by the exogenous application of iron. They also show, through promoter β -glucuronidase analysis and *in situ* hybridization, that *IRT1* is expressed in the external cell layers of the root, specifically in response to iron starvation. These results clearly demonstrate that *IRT1* is the major transporter responsible for high-affinity metal uptake under iron deficiency (Vert *et al.*, 2002).

Gross *et al.*, (2003) studied iron homeostasis related genes in rice and reported that *AtNRAMP1* was related to Fe sub cellular transport and its targeting to storage compartment such as vacuoles or plastids.

Takahashi, (2003) showed that some graminaceous plants are known to secrete high amounts of mugineic acid (MAs) family phytosiderophores under Fe-deficiency. This Fe acquisition system is called the Strategy-II mechanism. Tolerance to Fe-deficiency in graminaceous plants is thought to depend on the quantity of MAs secreted by plants under Fe-deficiency stress. This system was utilized to enhance the tolerance of rice to low Fe availability. Transgenic rice expressing the barley *naat* genes, one of the genes for the enzymes on the biosynthetic pathway of MAs, showed tolerance to low Fe availability when grown in a calcareous soil.

Mobilization of iron by plants is achieved by different strategies, either by secretion of plant-borne chelators or by reductive and proton promoted processes (Schmidt, 2003). These reactions, and subsequent uptake of Fe via specific transporters, are increased when the Fe requirements of the plant are not being met. When iron is taken up in excess of cellular needs, toxic oxygen radicals can form.

Gross *et al.*, (2003) analyzed a total of forty-three genes belonging to five known protein families: eighteen *YS* (Yellow Stripe), two *FRO* (Fe^{3+} -chelate reductase oxidase), thirteen *ZIP* (Zinc regulated transporter / Iron regulated transporter Protein), eight *NRAMP* (Natural Resistance - Associated Macrophage Protein), and two Ferritin proteins. The possible cellular localization and number of potential transmembrane domains were evaluated, and phylogenetic analysis performed for each gene family. Annotation of genomic sequences was performed. The presence and number of homologues in each gene family in rice and Arabidopsis is discussed in light of the established iron acquisition strategies used by each one of these two plants.

Yellow-stripe 1 like (*YSL*) transporters, that share sequence similarity with maize *YS1*, are likely involved in the transport of Fe (II)–NA complexes. The *YSL* genes in Strategy II plants also play a role in long distance Fe transport. The maize *YS1* gene is expressed both in roots and shoots. Several rice *YSL* genes (*OsYSL6*, *OsYSL14*, and *OsYSL16*) are expressed both in roots and shoots, *OsYSL2* and *OsYSL13* are expressed preferentially in shoots. More specifically, *OsYSL2* is expressed in the companion cells of the phloem and the vascular bundles in flowers and in developing seed. Together with the localization to the plasma membrane, *OsYSL2* is suggested to play a role in Fe (II)–NA transport in phloem and rice grains (Koike *et al.*, 2004).

Identification of the essential gene for Fe -deficiency induced Transcription Factor 1 (*FIT1*) which encodes a putative transcription factor that regulates iron uptake responses in *Arabidopsis thaliana* (Colangelo and Guerinot, 2004). Like the Fe (III) chelate reductase *FRO2* and high affinity Fe (II) transporter *IRT1*, *FIT1* mRNA is detected in the outer cell layers of the root and accumulates in response to iron deficiency. *FIT1* mutant plants are chlorotic and die as seedlings but can be rescued by the addition of supplemental iron, pointing to a defect in iron uptake. *FIT1* mutant plants accumulate less iron than wild-type plants in root and shoot tissues. Microarray analysis shows that expression of many (72 of 179) iron-regulated genes are dependent on *FIT1*. Demonstrate *FIT1* regulates *FRO2* at the level of mRNA accumulation and *IRT1* at the level of protein accumulation. They propose a new model for iron uptake in Arabidopsis where *FRO2* and *IRT1* are differentially regulated by *FIT1*.

Koike *et al.*, (2004) identified 18 putative yellow stripe 1 (*YS1*)-like genes (*OsYSLs*) in the rice genome that exhibited 36–76% sequence similarity to maize iron(III)-phytosiderophore transporter *YS1*. Of particular interest was *OsYSL2*, the transcripts of which were not detected in the roots of either iron-sufficient or iron-deficient plants, but dramatic expression was induced in the leaves by iron deficiency. Based on the nucleotide sequence, *OsYSL2* was predicted to encode a polypeptide of 674 amino acids containing 14 putative transmembrane domains. In iron-deficient leaves, the *OsYSL2* promoter was active in all tissues with particularly strong GUS activity evident in companion cells. The phloem-specific expression of the *OsYSL2* promoter suggests that *OsYSL2* is involved in the phloem transport of iron. Strong *OsYSL2* promoter activity was also detected in developing seeds. These results suggest that *OsYSL2* is a rice metal-NA transporter that is responsible for the phloem transport of iron and manganese, including the translocation of iron and manganese into the grain.

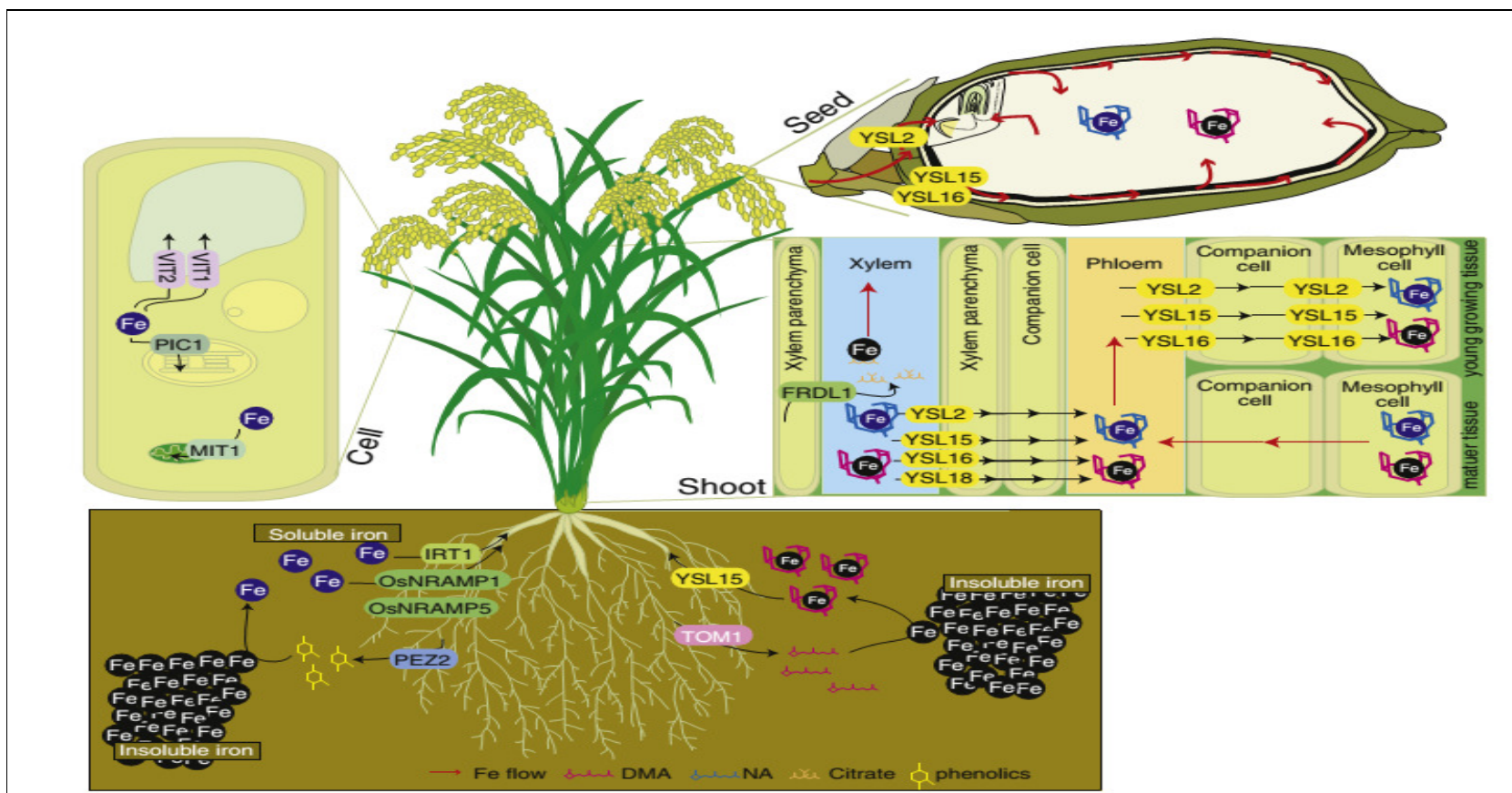


Figure: 1 Iron transport in rice from root to grains. Bashir *et al.*, (2013)

Ishimaru *et al.*, (2006) isolated the *OsIRT2* gene from rice, which is highly homologous to *OsIRT1*. Real-time PCR analysis revealed that *OsIRT1* and *OsIRT2* are expressed predominantly in roots, and these transporters are induced by low-Fe conditions. *OsIRT1* promoter–GUS analysis revealed that *OsIRT1* is expressed in the epidermis and exodermis of the elongating zone and in the inner layer of the cortex of the mature zone of Fe-deficient roots. *OsIRT1* expression was also detected in the companion cells. Analysis using the positron-emitting tracer imaging system showed that rice plants are able to take up both an Fe^{3+} -phytosiderophore and Fe^{2+} . This result indicates that, in addition to absorbing an Fe^{3+} -phytosiderophore, rice possesses a novel Fe-uptake system that directly absorbs the Fe^{2+} , a strategy that is advantageous for growth in submerged conditions.

A putative basic helix–loop–helix (bHLH) transcription factor gene, named *OsIRO2*, was strongly expressed in both roots and shoots during Fe deficiency stress. The expression of *OsIRO2* was induced exclusively by Fe deficiency, and not by deficiencies in other metals. Expression of the barley *HvIRO2* gene, which is a homologue of *OsIRO2*, was also induced by Fe deficiency (Ogo *et al.*, 2006).

The mechanism of Fe uptake and translocation in rice is of utmost importance in the development of rice varieties that are tolerant to low Fe availability and with high seed levels of Fe. In recent years, the mechanisms underlying Fe transport and homeostasis have been revealed, providing opportunities to increase the Fe content of rice grain. As excess Fe is toxic to cells, plants have developed sophisticated mechanisms to control Fe flow, making it difficult to alter Fe transport. Thus, choosing appropriate chelators and Fe transporters driven by appropriate promoters seems to be the key in developing rice that is tolerant to low Fe availability and which accumulates high grain levels of Fe (Bashir *et al.*, 2013). Many recent studies have been aimed at increasing the Fe content of rice.

Under Fe-deficient conditions, rice secretes a metal-cation chelator called deoxymugineic acid (DMA) to acquire Fe from the soil. In addition, DMA and its intermediate nicotianamine (NA) are thought to be involved in long distance Fe transport in rice (Nozoye *et al.*, 2007). Using promoter– β -glucuronidase (GUS) analysis, they investigated the expression patterns during seed germination of the Fe deficiency-inducible genes *OsNAS1*, *OsNAS2*, *OsNAS3*, *OsNAAT1*, and *OsDMAS1*, which encode enzymes that participate in the biosynthesis of DMA, and the transporter genes *OsYSL2* and *OsIRT1*, which are involved in Fe transport. All of these genes were expressed in germinating seeds prior to protrusion of the radicle. These results suggest that DMA and NA are produced and involved in Fe transport during germination.

Ogo *et al.*, (2007) characterized the Fe-deficiency-inducible basic helix-loop-helix transcription factor *OsIRO2* in rice. In rice, overexpression of *OsIRO2* resulted in increased MAs secretion, whereas repression of *OsIRO2* resulted in lower MAs secretion and hypersensitivity to Fe deficiency. Northern blots revealed that the expression of the genes involved in the Fe (III)–MAs transport system was dependent on *OsIRO2*. The expression of the genes for nicotianamine synthase, a key enzyme in MAs synthesis, was

notably affected by the level of *OsIRO2* expression. Microarray analysis demonstrated that *OsIRO2* regulates 59 Fe-deficiency-induced genes in roots. Some of these genes, including two transcription factors upregulated by Fe deficiency, possessed the *OsIRO2* binding sequence in their upstream regions. *OsIRO2* possesses a homologous sequence of the Fe-deficiency-responsive cis-acting elements (IDEs) in its upstream region.

Expression of *OsYSL15* in root epidermis and stele was induced by iron deficiency and showed daily fluctuation. *OsYSL15* promoter β -glucuronidase analysis revealed that *OsYSL15* expression in roots was dominant in the epidermis or exodermis and phloem cells under conditions of iron deficiency and was detected only in phloem under iron sufficiency (Inoue *et al.*, 2008). These results strongly suggest that *OsYSL15* is the dominant iron (III)-deoxymugineic acid transporter responsible for iron uptake from the rhizosphere and is also responsible for phloem transport of iron. *OsYSL15* was also expressed in flowers, developing seeds, and in the embryonic scutellar epithelial cells during seed germination. These results demonstrate that rice *OsYSL15* plays a crucial role in iron homeostasis during the early stages of growth.

Nicotianamine, the precursor of phytosiderophores, chelates Fe^{2+} and Zn^{2+} and plays an important role in transporting these metals to both vegetative and reproductive organs within the plant. They increase the Fe and Zn contents in rice grains by overexpressing the barley nicotianamine synthase gene *HvNAS1*. *HvNAS1*-overexpressing transgenic rice showed increased *HvNAS1* expression and subsequent increases in endogenous nicotianamine and phytosiderophore content in shoots, roots, and seeds. Fe and Zn concentrations in polished T1 seeds from transgenic plants increased more than two to three fold, respectively. Similarly, Fe and Zn concentrations also increased in both polished and brown T2 seeds (Masuda *et al.*, 2009). These results suggest that the overproduction of nicotianamine enhances the translocation of Fe and Zn into rice grains.

Masuda *et al.*, (2009) reported that mugineic acid family phytosiderophore play a very important role Fe uptake from the soil and its transport in Gramineous plants. Hypothesis suggests the possibility of enhancing Fe uptake and translocation for Fe fortification in the seed by introducing barley *HVNAS*, *HVNAAT-A*, *HVNAAT-b* and *IDS-3* in to the rice genome. The Fe and Zn concentration in seeds were higher than in the other transgenic and non-transgenic lines, these results suggests that the enhanced expression of MAs biosynthetic genes produces rice seeds with higher Fe (1.25)and Zn (1.30) content.

Among the 18 YSL genes in rice (*OsYSLs*), *OsYSL18* is predicted to encode a polypeptide of 679 amino acids containing 13 putative transmembrane domains. Reverse transcriptase PCR analysis revealed more *OsYSL18* transcripts in flowers than in shoots or roots. *OsYSL18* promoter β - glucuronidase (GUS) analysis revealed that *OsYSL18* was expressed in reproductive organs including the pollen tube. In vegetative organs, *OsYSL18* was specifically expressed in lamina joints, the inner cortex of crown roots, and phloem parenchyma and companion cells at the basal part of every leaf sheath (Aoyama

et al., 2009). These results suggest that *OsYSL18* is an iron phytosiderophore transporter involved in the translocation of iron in reproductive organs and phloem in joints

Lee *et al.*, (2009) used the nicotianamine synthase (*NAS*) gene to increase mineral contents in rice grains. Nicotianamine (NA) is a chelator of metals and a key component of metal homeostasis. They isolated activation-tagged mutant lines in which expression of a rice *NAS* gene, *OsNAS3*, was increased by introducing 35S enhancer elements. Shoots and roots of the *OsNAS3* activation tagged plants (*OsNAS3-D1*) accumulated more Fe and Zn. Seeds from *OsNAS3-D1* plants grown on a paddy field contained elevated amounts of Fe (2.9-fold), Zn (2.2-fold), and Cu (1.7-fold). Stein *et al.*, (2009) were able to analyze the expression profile of the two rice ferritin genes (*OsFER1* and *OsFER2*). Both genes are expressed, although with different regulation and organ distribution. Exposure to copper, paraquat, SNP and excess iron led to accumulation of ferritin mRNA, remarkably of *OsFER2*. *OsFER2* mRNA levels in rice flag leaves and panicles at different reproductive stages were higher than *OsFER1* mRNA levels. No ferritin mRNA was detected in rice seeds. However, imbibition under light led to ferritin expression, which was abolished when seeds were kept in the dark, suggesting a light-regulated induction. Ferritin mRNA accumulation was seen in the dark only when seeds were germinated in the presence of externally supplied iron. They suggest that the primary role of rice ferritin is related to defense against iron-mediated oxidative stress.

Jeong and Connolly (2009) reported that FROs reduce ferric iron chelates to form soluble ferrous iron. The founding member of the family, Arabidopsis *FRO2*, reduces iron at the root surface so that it may be transported across the plasma membrane. In particular, *FRO7* plays an essential role in iron delivery to chloroplasts, while two other *FROs* (*FRO3* and *FRO8*) localize to the mitochondria and might therefore contribute to mitochondrial iron homeostasis.

Lee *et al.*, (2009) characterized *OsYSL15*, one of the rice (*Oryza sativa*) YSL-like (*YSL*) genes that are strongly induced by iron (Fe) deficiency. The *OsYSL15* promoter fusion to β -glucuronidase showed that it was expressed in all root tissues when Fe was limited. In low-Fe leaves, the promoter became active in all tissues except epidermal cells. This activity was also detected in flowers and seeds. The *OsYSL15* green fluorescent protein fusion was localized to the plasma membrane. *OsYSL15* functionally complemented yeast strains defective in Fe uptake on media containing Fe^{3+} -deoxymugineic acid and Fe^{2+} -nicotianamine. Overexpression of *OsYSL15* increased the Fe concentration in leaves and seeds from transgenic plants. Altogether, these results demonstrate roles for *OsYSL15* in Fe uptake and distribution in rice plants.

Chandel *et al.*, (2010) characterized root transcriptome of diverse indica rice cultivars for expression of ten known metal homeostasis related genes in plants grown under controlled condition [with Fe (III)-HEDTA iron source]. Expression analysis showed expression of *OsFRO2*, *OsZIP9*, *OsYSL3*, *OsIRT1* and *OsZIP5* in most of the cultivars. The cDNA amplicons of *OsFRO2* and *OsZIP9* from different cultivars were analysed for sequence homology and several variation in their nucleotide sequences among rice genotypes. More than 94% sequence homology was observed for both the

genes. They analysed the genomic region underlying these genes to obtain information about possible spatial localization based on overlapping ESTs and MPSS tags.

Banerjee *et al.*, (2010) were analyzed variability for grain protein and Fe/Zn contents and factors affecting them for identification of rice genotypes with higher nutritive value wide variation for grain protein and micronutrient levels were recorded among the tested rice genotypes, which ranged from 6.19 to 10.75% for grain protein content, 4.82 to 22.69 µg/g for grain Fe and 13.95 to 41.73 µg/g for grain Zn content. Three genotypes, R-RF-31, Lalmati and R 1033-968-2-1 were identified as high protein and Fe/Zn containing rice lines.

Kim and Guerinot (2007) reported that iron uptake in plants is highly regulated in order to supply amounts sufficient for optimal growth while preventing excess accumulation. In response to iron deficiency, plants induce either reduction-based or chelation-based mechanisms to enhance iron uptake from the soil. New information has emerged on transporters that play a role in xylem loading and phloem loading/unloading of iron, and on the iron chelators involved in iron homeostasis. Some of the components regulating iron deficiency responses also have been elucidated, demonstrating that iron dependent gene regulation occurs at both the transcriptional and post-transcriptional levels.

Differential expression of metal homeostasis related candidate genes was observed among genotypes, tissue types and developmental stages. Maximum number of genes (twenty four) genes expressed in flag leaf tissue (MGF) showing variation in level of expression among genotypes (Banerjee and Chandel, 2011). Root transcriptome profiling revealed that nine genes (*OsZIP4*, *OsZIP11*, *OsNRAMP5*, *OsNRAMP7*, *OsYSL2*, *OsYSL4*, *OsYSL6*, *OsYSL9*, *OsNAAT1* and *OsNAC*) expressed at both developmental stages (MGF and MTS), three genes (*OsFER1*, *OsVIT1* and *OsFRO2*) expressed at MTS only while seven genes (*OsIRT1*, *OsFER2*, *OsZIP7*, *OsZIP8*, *OsZIP9*, *OsNRAMP4*, *OsNRAMP6* and *OsYSL12*) expressed at MGF stage only. Characterization of rice leaf transcriptome at MTS revealed negligible or poor level of expression of eleven genes (*OsFER2*, *OsIRT1*, *OsNAS2*, *OsYSL12*, *OsYSL17*, *OsNRAMP4*, *OsNRAMP6*, *OsZIP10*, *OsZIP5*, and *OsZIP9*), uniform high level of expression of four genes (*OsYSL4*, *OsNRAMP5*, *OsNRAMP7* and *OsZIP8*) and variation in level of expression of rest of the fifteen genes. Out of the total metal homeostasis related candidate genes expressed in flag leaf tissue five genes (*OsNAC*, *OsIRT1*, *OsZIP4*, *OsNAS2* and *OsYSL2*) expressed at uniform high level in all rice genotypes and *OsFER2* expressed only in flag leaf tissue. Expression of nine genes (*OsFER1*, *OsNRAMP4*, *OsNRAMP5*, *OsNRAMP6*, *OsYSL6*, *OsYSL12*, *OsYSL4*, *OsZIP8*, *OsZIP10*) was found to be correlated to high grain Fe contents, seven genes (*OsNAC*, *OsYSL2*, *OsYSL9*, *OsZIP4*, *OsVIT1*, *OsNAAT1*, *OsNRAMP7*) to high grain Zn contents whereas expression of two genes (*OsFRO2*, *OsZIP7*) was found to be correlated to both high grain Fe and Zn contents.

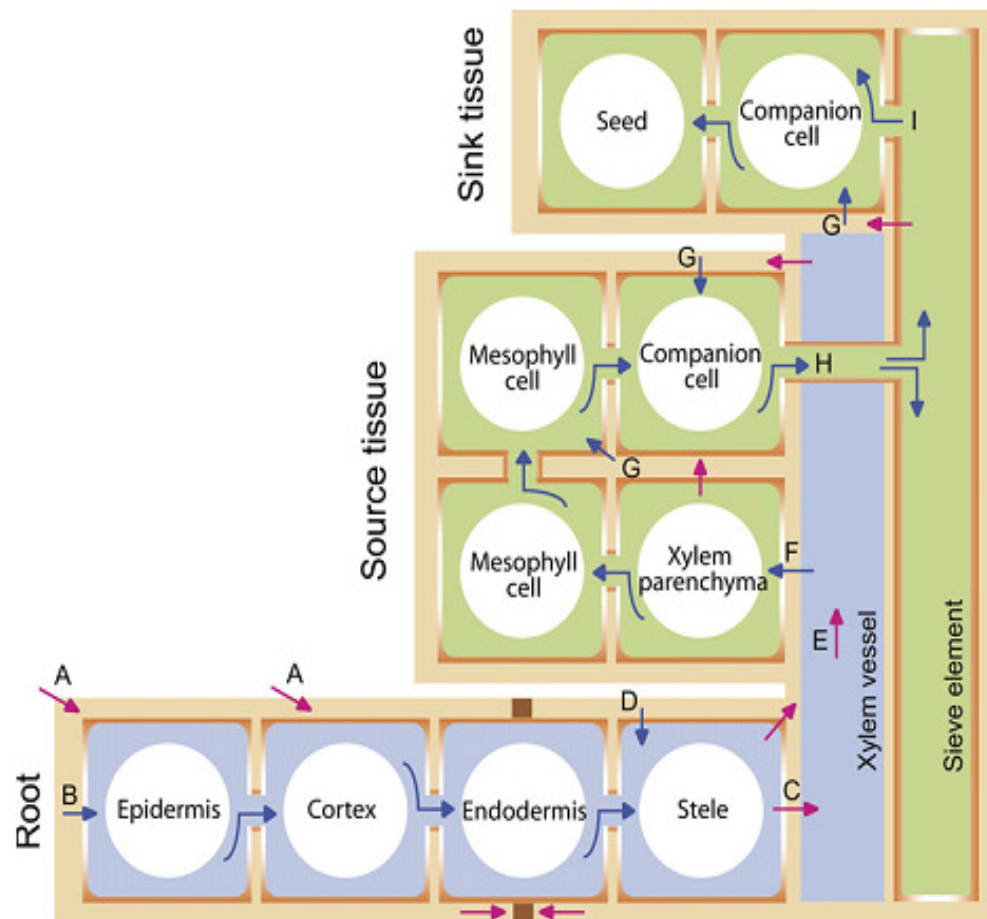


Figure 2: Iron transport from soil to seed. (Kim *et al.*, 2007)

2. Aerobic Rice

Rice is a semi-aquatic cereal crop grown in diverse climatic conditions. It is one of the most water consuming crops which receive an estimated 34 – 43 % of the total world's irrigation water, or 24 – 30 % of the total world's freshwater withdrawals (Bouman *et al.*, 2007). Asia is the major rice producer which accounts for an estimated production of 90 % of the world rice crop (Huke and Huke, 1997).

Aerobic rice is a new method of cultivating rice in less water than traditional flooded condition. Aerobic rice varieties are developed by crossing lowland varieties with upland varieties and cultivated in irrigated but non flooded and non-puddle soils (Bouman *et al.*, 2002; Bouman *et al.*, 2005).

Atlin *et al.*, (2004) noticed that both irrigated high yielding varieties and aerobic types yielded approximately four tonnes per hectare under low-land conditions, in the non-stress upland environment. Aerobic rice varieties significantly out yielded all other types and recorded significantly more panicles than traditional upland varieties with higher water use efficiency compared to normal method of planting.

A group of first-generation elite aerobic rice varieties such as "Han Dao 297," "Han Dao 277," "Han Dao 502," "Han 58," "Danjing 5," and "Danjing 8" has been developed and released in northern China (Wang *et al.*, 2002). The cultivation of aerobic rice aims to minimize the water requirement of rice while maintaining yield levels close to the irrigated lowlands. Traditionally, aerobic rice is grown in fragile rainfed uplands with low or no inputs and uncertain water availability. As fresh water is becoming scarce, aerobic rice cultivation is expected to expand to the regions of more intense cropping with high external inputs and productivity (Lafitte *et al.*, 2002).

Bouman *et al.*, (2002) compared aerobic rice varieties with lowland rice. Highest recorded aerobic rice yields were 4.7-6.6 t ha⁻¹, compared to 8-8.8 t ha⁻¹ of lowland rice, and reported aerobic rice variety Han Dao 502 is most promising because of its relatively high yield under both aerobic and flooded conditions and because of its good quality fetching a high market price. And his experiments showed that water inputs in aerobic rice were more than 50% lower (only 470-650 mm), water productivities 64-88% higher, gross returns 28-44% lower (345-633 \$ ha⁻¹) and labour use 55% lower due to its low water use.

Rainfed lowland rice grows in bunded fields that are flooded for at least part of the cropping season to water depths that do not exceed 50 cm for more than 10 consecutive days (Singh *et al.*, 2005).

The sustainability of food production and ecosystem services of rice fields are threatened by increasing water scarcity. Therefore, there is a need to develop and disseminate water management practices that can help farmers to cope with water scarcity. One of the long term solutions to cope with this problem is the development of "aerobic rice."

BI33 (ARB6) is an aerobic adapted high yielding rice variety developed from a cross between Budha and IR64 in University of Agricultural Sciences, GKVK, Bangalore (www.aerobicrice.in). It has manifested high degree of drought resistance and give high yield, comparable to improved varieties when sufficient moisture is available. It has deep roots which are capable of extracting moisture from deeper layers of soil column (Gowda 2011). It grows fast and has high biomass.

Aerobic rice has efficient water use efficiency which saves about 45% of water utilization, decrease methane production, reduce production cost and eco-friendly compared to conventional irrigated rice (Shashidhar, 2007).

A reduction in sink size associated with a dramatically reduced spikelet number per unit land area (20000-24000/m²) was identified as the major limitation of aerobic cultivation. By reducing water use during land preparation and limiting seepage, percolation, and evaporation, aerobic rice had about 51% lower total water use and 32-88% higher water productivity, expressed as gram of grain per kilogram of water, than flooded rice (Bouman *et al.*, 2005). Here it is important to note that the study was not done with varieties specifically adapted to aerobic method of cultivation, not available at that point in time.

Vijaykumar *et al.*, (2006) studied over 2600 improved germplasm lines, including 600 drought tolerant introgression lines, 800 *indica*/tropical *japonica* derivatives and 800 hybrid rice (*Oryza sativa* L.) breeding materials were screened in a puddled field (clay soil) under aerobic (non-flooded, irrigated) conditions with Rasi and Vandana as check varieties. The checks, known for their water stress tolerance were planted at regular intervals (after every ten entries) to serve as controls. In both the checks, within as well as between the strips, there was no variation for days to heading, whereas a significant variation was found for grain yield. Apart from visual selection, three different methods utilizing appropriate statistics were employed for identifying promising genotypes.

Special aerobic rice varieties called “Han Dao” (Such as HD 297, HD277, and HD502) have been developed by the China Agricultural University and are widely grown by farmers. In tropical Asia, IRRI has recently initiated its work on tropical aerobic rice systems and identified that a lowland variety “Magat” and some upland genotypes like “APO” and “CT 6510-24-1-2” perform well under aerobic conditions (Amudha *et al.*, 2009).

Verulkar *et al.*, (2010) identified breeding lines based on high yield potential with drought tolerance by employing direct selection for grain yield under drought stress, and testing their performance in rainy season managed-drought screenings (RSMDS) of different stress severity levels at several locations during the main season in rainfed rice production areas in India. These promising breeding lines out yielded the widely grown controls by about 0.5 tha⁻¹ under moderate stress and 0.7–1.0 tha⁻¹ under severe stress. Their results indicate that managed stress screening for drought tolerance, with a focus on selection for yield under stress, is effective in generating drought-tolerant cultivars.

Aerobic rice saved 73% of irrigation water for land preparation and 56% during the crop growth period. Aerobic rice with micro irrigation practices leads sustainable rice production methodology for immediate future to address water scarcity. This technology is better remedy for future climate change under drought condition with lesser greenhouse gas (GHG) emission. Selection of good aerobic rice variety with desired physiological attributes gives better performance (Parthasarathi *et al.*, 2012).

3. Biofortification

Biofortification is a combined approach of conventional plant breeding and modern biotechnology to enrich vital nutrients (minerals, vitamins and proteins) in staple crops (Graham *et al.*, 1999; Pfeiffer and McClafferty, 2007).

Welch and Graham (2004) found 4-fold differences in mineral levels of iron and zinc concentration among rice genotypes, which suggests that there is a potential to increase the concentration of these micronutrients in rice grain with genetic technology.

Realizing the importance of Zn for human beings, there are several approaches undertaken by researchers to enhance its content in grains of plants (Gregorio *et al.*, 1999; Lu *et al.*, 2008).

The best candidate genotypes selected for biofortification programme should fulfill the criteria of high productivity; show considerable health impact for micronutrient enrichment levels; the trait for micronutrient must be relatively stable across various environmental conditions and climatic zones and consumer acceptance for taste and cooking quality (Goto, 1999; Gregorio, 1999; Welch and Graham 2001; Welch and Graham; *et al.*, 2004).

Zn food fortification and supplementation approaches are expensive and not easily accessible for people living in developing countries (Stein *et al.*, 2007; Pfeiffer and McClafferty, 2007).

Biofortification under HarvestPlus program are underway for six staple foods: rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), cassava (*Manihot esculenta* Crantz), orange-fleshed sweet potato [*Ipomoea batatas* (L.) Lam.], and common beans (*Phaseolus vulgaris* L.) that are consumed by the majority of the world's poor in Africa, Asia, and Latin America (Pfeiffer and McClafferty, 2007).

Breeding staple cereal crops dense in minerals is a low-cost, sustainable strategy to ameliorate micronutrient malnutrition for people living in developing countries which cannot afford pulses, fruits, vegetables, fish and animal products enriched with micronutrients in the diet (Martinez *et al.*, 2007; Cakmak, 2008).

According to HarvestPlus Challenge Program MTP 2009-2011 project portfolio, breeding target levels for high zinc rice is a minimum increment of 8 micrograms zinc/gram to a base line of 16 micrograms zinc/gram in polished rice to get a considerable biological impact on human health.

Three QTLs responsible for zinc content (qZN-5, qZN-7 and qZN-11), located on chromosome number 5, 7 and 11 respectively (Chandel, *et al.*, 2011).

3.1 Candidate gene

Candidate genes (CG) are sequenced genes of known biological function associated with the manifestation of the trait. They may be structural genes or genes in a regulatory or biochemical pathway which affect trait expression. One hypothesis states that “a significant proportion of the quantitative trait loci (QTL) affecting trait variation are in fact CGs associated with that trait” (Rothschild and Scoller, 1997).

Candidate genes are sequenced genes of known biological action involved in the development or physiology of a trait (Vinod *et al.*, 2006). Candidate genes for economically important traits have been potentially useful in plant breeding. Candidate genes have been successfully used in genetic and association mapping, MAS and development of transgenic plants for various traits in crop plants.

4. Variability, Heritability, Genetic advance

Genotypic variation for micronutrient accumulation in grain have been reported in staple crops such as rice (Graham *et al.*, 1999; Gregorio *et al.*, 1999; Gregorio *et al.*, 2000; Zhang *et al.*, 2004), wheat (Ortiz-Monasterio and Graham, 2000) and soybean (Raboy *et al.*, 1984).

There is a large genetic variation for grain Zn content in rice germplasm accessions and this variation can be exploited in breeding programs to enhance Zn content in the grains (Graham *et al.*, 1999; Welch and Graham, 2004).

Rice is not considered as a major mineral supplier, but any increase in mineral concentration could significantly help reduce the iron and zinc deficiency problems among target populations.

Graham *et al.* (1999) reported the presence of genetic variation for grain Zn concentration among 1000 rice genotypes grown and screened in International Rice Research Institute (IRRI) farm, Los Banos, Philippines, where its concentration in the brown rice ranged from 15.3 to 58.4 ppm while Fe concentration ranged from 6.3 to 24.4 ppm.

A trait for high iron and zinc has been linked to aromatic varieties such as jasmine and basmati (Graham *et al.*, 2001).

One of the best rice lines developed by IRRI, designated IR68144-3B-2-2-3, contains 21 ppm of iron and 34 ppm of zinc obtained from a cross between high yielding variety (IR72) and a tall, traditional variety (Zawa Bonday) from India. This elite line has good grain quality, high yielding, and preliminary studies show that it improves human nutrition (Bouis, 2001).

Nine genotypes of rice were evaluated for iron and zinc content in rice grain at IRRI by Martinez *et al.* (2006), found a range of 8.8 to 21.0 ppm, 14.0 to 40.0 ppm for iron, and zinc respectively.

Genetic diversity studies for eight mineral concentrations of brown rice, using 653 accessions showed that there is greater average genetic diversity index for japonica accessions compared to indica accessions (Zeng *et al.*, 2005).

Grain Zn concentration is substantially higher in certain landraces of Southeast Asia than in commonly grown high yielding rice varieties (Zozali *et al.*, 2006).

Singh *et al.*, (2010) reported Zn content in grains of rice ranges from 30 ppm in Selection New 2 to 64 ppm in Dular among 25 rice genotypes taken for the study.

Nagarathna *et al.*, (2010) reported the presence of wide genetic variability in rice Zn content from 0.84 to 5.00 mg/100g dry weight.

Martínez *et al.*, (2010) evaluated 11,400 rice samples collected in local stores and supermarkets in Colombia, Bolivia, Nicaragua and the Dominican Republic for iron and zinc content during 2006 - 2009 in brown and milled rice samples found 2 – 3 ppm for iron and 16 – 17 ppm for zinc in milled rice, whereas values for brown rice were 10 – 11 ppm for iron and 20 – 25 ppm for zinc.

The contents of Fe and Zn in grains of traditional rice genotypes were significantly higher than those of improved cultivars (Anandan *et al.*, 2011).

On the basis of grain zinc content, rice genotypes could be grouped into three categories, low (less than 14 ppm), moderate (14 ppm – 24 ppm) and high (greater than 25 ppm).

Total variability of a trait is divided into genotypic variability and phenotypic variability. The estimate of variability suggests the variation in heritable portion of a trait that could be transferred from parent to offspring in response to selection (Hallauer and Carena, 2009; Lakshmana *et al.*, 2009).

Heritability in broad sense was proposed by Hanson *et al.*, (1956) as the ratio of genetic variance to the total variance, while the narrow sense heritability has been defined as the ratio of additive variance to the total variance (Lush, 1949).

According to Falconer (1960) the higher the genotypic to phenotypic variation ratio indicated the more heritability of the trait and the smaller the ratio showed the presence of higher influence of the environment on the phenotypic expression of the trait.

Genetic coefficient of variability along with heritability gave an idea of expected genetic gain from selection. Heritability value indicates the relative effectiveness of selection based on phenotypic expression of a trait but genetic advance is more useful in predicting the actual value of selection (Johnson *et al.*, 1955).

Graham *et al.*, (1999) noticed higher iron content trait expressed in all rice environments analysed in dry season in normal and saline soils, in acid and neutral soils. Grain iron content generally varied much more with genotype, than with environment and genotype x environment interactions.

Ortiz-Monasterio *et al.*, (2000) at International Maize and Wheat Improvement Center (CIMMYT) reported a significant genotype X environment interactions for Fe and Zn grain concentrations in wheat where there is a strong genetic component for Fe and Zn accumulation in the grain.

Populations which are genetically more uniform are expected to show lower heritability than genetically diverse populations. Since environmental variance forms part of phenotypic variance, it affects the magnitude of heritability.

Kibanda and Luzi-kihupi (2007) reported absence of environmental influence on grain length and grain size (length/width) but observed higher genetic variance for these traits.

Studies by HarvestPlus showed that there is a significant $G \times E$ interaction in the expression of iron and zinc in the grain. Experiments carried out in Colombia, the Dominican Republic, and Nicaragua reported similar results. In grain Zn content, $G \times E$ and $G \times \text{soil Zn}$ interactions showed significance (Pandian *et al.*, 2011).

Venuprasad *et al.*, (2002) reported high phenotypic and genotypic coefficient of variation for number of tillers per plant, grain yield per plant, total biomass per plant, harvest index.

Inheritance studies indicated that rice grain length is highly heritable (Mackill *et al.*, 1995).

El-Malky *et al.*, (2008) observed high broad sense heritability estimate of 98.89 % for days to maturity, 75.20 % for the number of tillers per plant, 41.74% for the number of panicles per plant, 98.97 % for 1000 grain weight.

Zhang *et al.*, (2005) reported high heritabilities for 100 grain weight, width and shape and moderate for grain length in a study done on six varieties of black rice and one variety of aromatic white rice.

Shukla *et al.*, (2005) revealed high co-efficient of variation as well as high values of heritability coupled with expected genetic advance for grain yield per plant, harvest index and biological yield per plant.

Genetic variability and heritability parameters were assessed in 114 genotypes and three checks by Suman *et al.*, (2005). High values of heritability coupled with high genetic advance as per cent of mean were observed for total number of tillers per plant, productive tillers per plant, number of filled grains per panicle, plant yield, biological yield and harvest index.

Courtney *et al.*, (2008) reported high broad-sense heritability for iron and zinc among full-sibling families of sweet potato (*Ipomoea batatas* (L.) Lam.) Suggesting traditional breeding strategies like mass selection could improve the micronutrient value.

Binse *et al.*, (2009) found high genotypic and phenotypic coefficient of variations for harvest index and 100 grain weight; high heritability coupled with high genetic advance for harvest index, grain yield per plant from a trial with four CMS lines, eight testers and thirty-two hybrids evaluated.

Garcia-Oliveira *et al.* (2009) reported medium to high heritability for microelements Fe, Zn, Mn, Cu, Ca, Mg, P and K with estimates of 72.8 %, 40.6 %, 55.9 %, 85.6 %, 70.6 %, 54.2 %, 46.4 % and 19.1 %, respectively in set of recombinant inbred lines of rice.

Susanto *et al.*, (Rice Genetic Conference, 2009) in IRRI reported that broad sense heritability of iron and zinc content in polished rice grains were 57.28 % and 76.36 % in population obtained from IR75862-206-2-8-3-B-B-B/IR64 parents.

Samak *et al.*, (2011) reported high genotypic and phenotypic coefficient of variations for grain Zn content in grains of rice. Samak *et al.*, (2011) reported the early fixation in genotypes for homozygosity for the concerned traits. It is found that the estimates of heritability and genetic advance of F₅ progenies for grain zinc and manganese content in rice showed subtle increase as compared to the F₄ progenies.

Akinwale *et al.*, (2011) in rice observed high to medium broad sense heritability for days to flowering, plant height; low broad sense heritability was observed for days to maturity, the number of tillers per plant and 1000 grain weight; high heritability and genetic advance were recorded for grain yield per plant.

Govindaraj *et al.*, (2011) found high value of heritability coupled with high genetic advance as per cent of mean for number of productive tillers (20.56 %), iron (75.27 %) and zinc (37.78 %) content in India's Pearl millet (*Pennisetum glaucum* (L) R. Br.) accessions.

5. DNA Markers and their linkage to grain zinc and iron content

Molecular markers augment conventional plant breeding for efficient and precise identification or selection of a trait of interest linked to them. Unlike morphological markers, these molecular markers are abundant, not influenced by environment and detected in all plant growth stages (Kumar, 1999; Collard *et al.*, 2005; Collard and Mackill, 2008; Moose and Mumm, 2008).

During the last few decades, molecular markers have been immensely used in plant biotechnology and their genetics studies. They are used in assessment of genetic variability and characterization of germplasm; estimation of genetic distance between population, inbreds and breeding material; genetic mapping; detection of monogenic and quantitative trait loci (QTLs); marker assisted selection; increase the speed and quality of backcrossing to introgress desirable traits from closely related varieties to elite germplasm; identification of sequences of useful candidate genes, etc (Farooq and Azam, 2002; Rana and Bhat, 2004; Murtaza *et al.*, 2005).

Some of the most commonly used molecular markers to assess the variability and diversity at molecular level are restriction fragment length polymorphism (RFLP; Botstein *et al.*, 1980), random amplified polymorphic DNA (RAPD; Williams *et al.*, 1990), simple sequence repeats (SSR; Akkaya *et al.*, 1992), Sequence characterized amplified regions (SCAR; Paran and Michelmore, 1993), ISSRs (Zietkiewicz *et al.*, 1994), amplified fragment length polymorphism (AFLP; Vos *et al.*, 1995) and single nucleotide polymorphism (SNP; Jordan and Humphries, 1994).

Microsatellites are tandem repeats of DNA sequences of only a few base pairs (1 - 6 bp) in length, the most abundant being dinucleotide repeats (Morgante and Olivieri, 1992). The completion of rice genome sequence provided an opportunity to identify literally tens of thousands of new targets for DNA markers, especially SSRs. There were 18,828 Class I (di-, tri-, tetra-repeats) SSRs released after the completion of the Nipponbare genome sequence in 2005 (Matsumoto *et al.*, 2005). It is estimated that the density of SSRs (approx. 51 SSRs per Mb) can provide a considerable map construction and MAS for numerous applications.

Sadeghzadeh *et al.* (2010) obtained one dominant microsatellite-anchored fragment length polymorphism (MFLP) marker SZnR1 (seed Zn regulator 1) associated with increased accumulation of Zn in barley seed in a study done using 150 double haploid mapping populations derived from a cross between Clipper (low-Zn-accumulator) and Sahara 3771 (high-Zn accumulator). It showed that SZnR1 marker was 12 cM from Xbcd175 marker on the short arm of chromosome 2H. This marker showed a correlation with seed Zn concentration and content and explained 21 and 18 % increase in seed Zn concentration and content, respectively.

Availability of molecular markers and understanding the genetic basis of accumulation of micronutrients in the grains has facilitated for mapping of the QTL and tag the genes responsible for the high zinc content and use these markers for devising the plant breeding strategies and for improving grain micronutrient content through marker-assisted selection (Zimmerman and Hurrel, 2002; Lu *et al.*, 2008; Tiwari *et al.*, 2009).

Zn accumulation in seeds involves a polygenic inheritance which is attributed by the interactions between two or more genes and their environment (Grusak and Dellapenna, 1999). QTLs responsible for the trait of interest can be identified with closely linked molecular markers.

Susanto *et al.*, (Rice Genetic Conference, 2009) in IRRI identified two QTLs controlling Zn content in polished rice on chromosome 6 (zn-vb6.1) and chromosome 12 (zn-vb12.1) and two QTLs controlling iron content on chromosome 3 (fe-vb3.1) and chromosome 6 (fe-vb6.1) from 115 BC₁F₁ population obtained from IR75862-206-2-8-3-B-B-B/IR64 parents.

Two QTLs controlling iron content in polished rice grains was identified. One QTLs on chromosome designated as fe-Vb31 (between M6931 and RM6832) and another on chromosome 6 designated as fe-Vb61 (RM276 and 8226) were identified (Susanto, 2009).

III. MATERIAL AND METHODS

The experiments were conducted related to this study are presented below.

- 1) Phenotypic characterization of zinc and iron content in grains, leaves, culm and roots of black rice and selected rice accessions under aerobic condition.
- 2) Molecular analysis of candidate gene markers associated with zinc and iron content in rice.

The details of the materials used, methods and protocols followed and statistical tools employed for analysis are presented under the respective experiments separately.

Experiment I:

3.1 Phenotypic studies of genotypes for rice grain, leaves, culm and root zinc and iron content and other yield related characters

3.1.1 Experimental site

The experiment was carried out during *Kharif* 2013 at Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore, India located at the latitude of 12° 58' North; longitude 77° 35' East and altitude of 930 meters above mean sea level (MSL).

3.1.2 Genetic materials

The genetic materials used in the present study consist of eight diverse genotypes. BI-33 is aerobic rice variety with high tillering and drought tolerance and Azucena is upland japonica variety, with tall stem, sparse tillering, low yielding and long grained aromatic variety. Jeerigesanna with small sized slender grains and Devamallige having very good taste and nutrition rich land race from Karnataka black rice with high anthocyanin grains are in black colour and very good nutritious with mostly grown in the eastern states. AM-65 and BJ21 (UAS Bangalore) were high micronutrient content lines with tall in plant height was nominated for all India trial. All the rice accessions selected were listed in Table 1.

3.1.3 Aerobic condition

Cultivation of rice in less water than traditional flooded condition. Aerobic rice varieties were developed by crossing lowland varieties with upland varieties and cultivated as a dry field crop in irrigated but non-flooded and non-puddle fertile soils (Bouman et al., 2005; Shashidhar, 2007).

3.1.4 Layout of experimental field

The experiment was laid out in Randomized Complete Block Design (RCBD) and eight genotypes with three replications and all the entries were allotted randomly using random number. Seeds were sown in the 6 inch diameter width and 60 cm length PVC pipes which were filled with soil and compacted with regular watering. Three seeds were sown in each pipe, after 15 days extra seedlings were thinned out from the pipes only one healthy seedling maintained and the field management was followed according to recommended packages of agronomic procedures of UAS, Bangalore to raise healthy crop. Irrigation was done once in three days for all the pipes. All necessary measures were taken to control pest and disease infestation.

Table 1: List of selected rice genotypes

SL. No	Genotypes	Characters
1	BI-33	Aerobic variety with high tillering and drought tolerance
2	Azucena	Aromatic, drought tolerant <i>joponica</i> variety
3	Jeerigesanna	Small slender grains and aromatic
4	Devamallige	Nutrition rich land race from Karnataka
5	IR-20	Medium slender grains with high yielding
6	Black rice	Forbidden rice, high Anthocyanin ,antioxidants content deep black color
7	AM-65	Cross between Azucena and Moro mutant, high zinc line
8	BJ-21	Cross between BI-33 and Jeerigesanna elite high zinc line

3.1.5 Recording of observations

The observation was taken for plant grown in PVC pipe. The replicated observations were recorded on these plants considered for analysis. The information about the characters observed are described as follows.

3.1.5.1 Plant height at maturity (cm)

The height of the plant from the base to the tip of the longest plant part was measured and expressed in cm.



Plate: 1 Plants at seedling stage



Plate: 2 Plants at maturity stage

3.1.5.2 Panicle length at maturity (cm)

The length of panicle was recorded in cm at the time of harvest.

3.1.5.3 Total Number of tillers per plant

Numbers of tillers (both productive and nonproductive) were counted at the time of harvest.

3.1.5.4 Number of productive tillers per plant

Numbers of productive tillers were counted at time of harvest.

3.1.5.5 Grain yield per plant (g)

The total weight of all filled grains of a plant was measured in grams.

3.1.5.6 Straw yield per plant (g)

Shoot dry weight was recorded after harvesting and threshing.

3.1.5.7 Root length (cm)

Root length was recorded at maturity.

3.1.5.8 Root numbers

Root numbers were counted manually after harvesting the root.

3.1.5.9 Root volume (cm³)

Root volume was recorded by using volume displacement method of water after harvesting of root.

3.1.5.10 Days to 50 per cent flowering

The number of days taken for each entry from sowing to opening of first flower in 50 per cent of the panicles was recorded.

3.1.5.11 Zinc and iron content in grains, leaves, culm and root (ppm)

Zinc and Iron content was estimated in the grains, leaves, culms and roots samples collected from the selected genotypes. Seeds of individual genotypes were harvested manually and hand dehusking was taken to avoid contamination. The leaf, culm and root samples were cut into small pieces by hand. The grains, leaves, culm and

root samples were washed quickly with double distilled water to remove any surface contaminants and dried in hot air oven at 70°C for 72 hours.

3.1.6 Digestion of the sample

Unbroken, uniform dried grains, leaves, culm and root samples of (0.5 g) were placed in the labeled clean conical flask. Then 5ml of concentrated nitric acid (70 per cent) was added and the mixture was kept for cold digestion for overnight. On the following day, 5ml of di-acid mixture {(Nitric acid: Perchloric acid in the ratio of 10:4, analytical reagent grade, (Merck))} was added to the cold digested sample. The mixture was then placed on the electric hot plate, allowing the evaporation of acid in the form of white fumes, until white precipitate was formed at the base of the flask. The digested sample was left to cool and the volume was then made up to 50 ml using double distilled water. The diluted solution was then filtered with Sonar® filter paper before being transferred to clean, airtight tubes and was kept ready for estimation of zinc and iron content. The content of zinc was measured using atomic absorption spectrometer (AAS, Thermo scientific) at 213.86 nm.

3.1.7 Statistical analysis

The mean values of three plants used for recording the observations were computed for different plant characters for each of the genotypes grown in *Kharif* 2013. The phenotypic data for all the genotypes for each character were subjected to statistical analysis. The statistical analysis of the data on individual characters was performed using WINDOSTAT 9.1 statistical package.

3.1.7.1 Analysis of variance

The analysis of variance for different characters was carried using the mean data for aerobic condition in order to partitioning the variance due to different sources following the method given by Panse and Sukhatme (1964).

Source of Variation	Degree of freedom	Sum of squares	Mean sum of squares	F-ratio
Replication	(r-1)	rSS		
Genotype	(g-1)	gSS	$\frac{gSS}{(g-1)} = eMS$	$\frac{gMS}{EMS}$
Error	(r-1)(g-1)	ESS	$\frac{ESS}{(r-1)(g-1)} = EMS$	
Total	(rg-1)	TSS		

Wherein,

r = Number of Replications

g = Number of Genotypes

The significance was tested by comparing with the table values as given by Fisher and Yates (1932). Standard error of means (SE_M) and Critical Difference (CD) was calculated using appropriate formula for comparing individual line means.

3.1.7.2 Estimation of genetic variability parameters

3.1.7.2.1 Phenotypic and genotypic variance

Phenotypic and genotypic components of variance were estimated by using the formula given by Cochran and Cox (1957).

$$\text{Genotypic variance } (\sigma_g^2) = \frac{\text{MSS due to genotypes} - \text{MSS due to error}}{\text{No. of blocks}}$$

$$\text{Phenotypic variance } (\sigma_p^2) = \text{Genotypic variance } (\sigma_g^2) + \text{MSS due to error}$$

3.1.7.2.2 Co-efficient of variability

Both phenotypic and genotypic co-efficient of variability for all the characters were estimated using the formulae of Burton and De Vane (1953).

Genotypic Co-efficient of Variability (GCV):

$$\text{GCV \%} = \frac{\sqrt{\text{Genotypic variance}}}{\text{Grand mean}} \times 100$$

Phenotypic Co- efficient of Variability (PCV):

$$\text{PCV \%} = \frac{\sqrt{\text{Phenotypic variance}}}{\text{Grand mean}} \times 100$$

PCV and GCV were classified as per Sivasubramanian and Menon (1973) as shown below:

Sl. No	Percentage Range	Class
1	0-10%	Low
2	11-20%	Moderate
3	>21%	High

3.1.7.2.3 Heritability in Broad sense (h^2)

Heritability (broad sense) was estimated for all the characters as the ratio of total genotypic variance to the total phenotypic variance as suggested by Hanson *et al.* (1956).

$$h^2 = \frac{\sigma_g^2}{\sigma_p^2}$$

According to Robinson (1966) heritability estimates in cultivated plants can be placed in following categorizes.

SL.No	Percentage Range	Class
1	0-30%	Low
2	30-60%	Moderate
3	> 60%	High

3.1.7.2.4 Genetic Advance (GA)

Genetic advance for each character was estimated by using the following formula of Johnson *et al.* (1955).

$$GA = H^2 \times K \times \sigma_p$$

Where,

H^2 = heritability estimate

K = selection differential which is equal to 2.06 at 5% intensity of selection.

σ_p = phenotypic standard deviation

3.1.7.2.5 Genetic advance as percent of mean

Further the genetic advance as per cent of mean was computed by using the following formula:

$$GA \text{ as per cent of mean} = \frac{GA}{\text{Grand mean}} \times 100$$

Genetic advance as per cent of mean was categorized according to Johnson *et al.* (1955), as given below

SL.No	Percentage Range	Class
1	0-10%	Low
2	11-20%	Moderate
3	> 21%	High

3.1.7.3 Correlation studies

Correlation coefficients measure the strength of association between two variables. The coefficient of correlation is the measure of association between two characteristics. Correlations can be either positive, when an increase in one variable is accompanied by an increase in another one, or negative when an increase in one character is accompanied by a decrease in another (Falconer, 1960).

3.1.7.3.1 Phenotypic correlations

It is the association between two characteristics that can be directly observed and determined from measurements of the two characteristics in individuals of the population (Falconer, 1960).

Phenotypic co-efficient of correlation between two characters were determined by using variance and covariance components as suggested by Al-Jibourie *et al.* (1958).

$$r_p(x,y) = \frac{p_x \cdot p_y}{\sqrt{V_{p_x} \cdot V_{p_y}}}$$

Where,

$p_x \cdot p_y$: phenotypic co-variance of x and y.

V_{p_x} : Phenotypic variance of x

V_{p_y} : Phenotypic variance of y

$r_{p(x,y)}$: phenotypic correlation co-efficient of x and y

The calculated value of 'r' was compared with table 'r' value from Fisher and Yates (1932) with n - 2 degree of freedom at 5 per cent and 1 per cent level of significance, where n refers to number of pairs of observations.

Experiment II

3.2 Molecular analysis of candidate gene markers associated with Zinc and Iron content in rice.

3.2.1 Genotyping

3.2.1.1 Leaf sample collection

The Plant material used for this study consisted of eight genotypes. Young healthy Leaves were collected from the field and placed in a plastic bag along with the tag identifying the sample name and number. The collected leaf samples were stored at -80°C until they were processed.

3.2.1.2 Isolation of Genomic DNA

DNA extraction for selected rice genotypes was done using CTAB method from young leaf tissues as the protocol described by Doyle and Doyle (1990) as below.

Requirements

- Leaf samples were collected from 21 days old seedlings and stored immediately at -70°C.
- Cetyl trimethyl ammonium bromide (CTAB) extraction (100ml)

CTAB	2% (w/v)
Tris HCl (pH 8.0)	100mm
Sodium Chloride	1.4M
EDTA	20mM

(Tris, sodium Chloride and EDTA were autoclaved and 2 % CTAB was added after autoclaving and preheated before using the buffer).

0.2 % β - Mercaptoethanol (added freshly).

Tris EDTA (TE) buffer

1M Tris HCl (pH 8.0)	10mM
0.5 M EDTA (pH 8.0)	1mM

(This was dissolved and made up to 100ml, autoclaved and stored at 4 °C)

- 2 % Polyvinyl poly pyrrolidone (PVP)
- Ice cold Isopropanol
- Chloroform: Isoamyl alcohol 24:1 (v/v)
- Sodium acetate (3.0 M, pH 7.0) (pH adjusted using glacial acetic acid)
- Absolute ethanol and 76 % ethanol (stored at -20 °C)
- RNase A- 10 mg/ml (RNase A was dissolved in TE buffer and stored at -20 °C).

Protocol for Extraction of genomic DNA

- Leaf samples (approximately 2g) were cut into small bits with sterile scissors and transferred to prechilled mortar.
- The leaf tissues were frozen using liquid nitrogen and ground to fine powder.
- The fine powder was allowed to thaw in 10ml of preheated extraction buffer in polypropylene centrifuge tubes and incubated for 30 minutes at 65 °C in water bath with occasional mixing.
- The tubes were removed from the water bath and equal volume of chloroform: Isoamyl alcohol mixture (24:1 v/v) was added and mixed by inversions for 15 minutes.
- The contents of polypropylene centrifuges tubes were centrifuged at 10000 rpm for 10 minutes at room temperature.
- The clear aqueous phase was transferred to a new sterile tube and equal volume of ice cold isopropanol was added and mixed gently by inversion and then kept in the freezer at 4 °C for the precipitation of DNA.
- Using blunt end tips, the precipitate was pooled out into a micro centrifuge tube and air dried after removing the supernatant by brief spin.

- 500 µl of chloroform: Isoamyl alcohol mixture was added and centrifuged at 10000 rpm for 10 minutes at room temperature.
- Aqueous phase was transferred to another microcentrifuge tube without disturbing the inner phase.
- To this, 2.5 volume of absolute alcohol and 1/10 volume of sodium acetate were added and kept for incubation overnight.
- It was centrifuged and the supernatant was discarded. To this 500 µl of each 70 % and 100 % ethanol was used subsequently to wash the DNA by centrifugation.
- The alcohol was discarded and DNA was completely air dried and DNA pellet was dissolved in 250 µl of TE and stored at 4 °C.

3.2.1.3 DNA Quality assay using Agarose Gel Electrophoresis

Materials

- 3X loading dye

5M NaOH	200 µl
95% formamide (v/v)	95 ml
50 % bromophenol blue (w/v)	50g
0.5 % xylene cyanol (w/v)	50mg

(Dissolved in sterile double distilled water and made up the volume to 100ml)

- 10X Tris Borate EDTA buffer (TBE)

Tris base	107.8 g
Boric acid	55.2g
EDTA (Na ₂ .2H ₂ O)	9.2g

(Dissolved in 800ml of sterile double distilled water and made up to 1000ml)

- 50X Tris Acetate EDTA buffer (TAE)

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA ($\text{Na}_2\cdot 2\text{H}_2\text{O}$) (0.5M, pH 8.0) 18.6 g in 100 ml of double distilled water	
(Dissolved in 800ml of sterile water and made up to 1000ml)	

- 100bp Ladder

40 μl 6X loading dye

10 μl 100 bp ladder

120 μl sterilized double distilled water

(Total volume made up to 170 μl)

Protocol

- The pyrex gel casting plate's open ends were sealed with cello tape and the comb was placed properly in casting plate kept on a perfectly horizontal platform
- 0.8 % (0.8 g/100ml) agarose was added to 1X TBE, boiled until the agarose dissolved completely and then allowed to cool. Ethidium bromide (DNA intercalating agent) was added when temperature reaches to 55 - 60 °C as the staining agent.
- Then it was poured into the gel mould with combs placed and allowed to solidify. The comb and the cello tape were removed carefully after solidification of the agarose.
- The casted gel was placed in the electrophoresis unit with wells towards the cathode and submerged with 1X TBE to a depth of about 1cm.

3.2.1.4 Loading the DNA samples

- 1 μl of the crude DNA sample was pipetted onto a parafilm and mixed well with 3 μl of 3X loading dye by pipetting up and down gently several times.
- The mixed contents were transferred to the wells of agarose gel placed inside the 1X TBE buffer.

3.2.1.5 Quantification of DNA

The genomic DNA was quantified using Nano drop (Biotek Epoch at 260 nm and 280 nm wavelengths. The absorbance at 260 nm allows the calculation of DNA concentration in the sample. An OD of 1 at 260 nm corresponds to 50 µg of double stranded DNA. A pure sample of DNA shows the ratio of OD 260/280 as 1.8. Ratios less than 1.8 indicate contamination in the isolation either with phenol or with proteins. The values higher than this indicate the presence of RNA in the isolation.

3.2.1.6 Normalization of the DNA concentration

Normalization of the DNA concentration was done to bring all the DNA concentrations to a relatively equal level (25 ng/µl) by appropriate dilutions for PCR reaction. Dilution was done with double distilled sterile water.

3.2.1.7 Screening of Candidate gene markers

14 Zinc (Table 2) and 15 Iron (Table 3) candidate gene markers were used for screening of Zinc and Iron content in rice.

Amplification condition

The amplification profile was as follows:

- | | | |
|--|--------|--------------|
| a. Initial denaturation temperature | 94°C | 5 min. |
| b. Denaturation | 94°C | 45 sec |
| c. Primer annealing | 55°C * | 1 min |
| d. Primer extension | 72°C | 45 sec |
| e. Later, step b) to d) was repeated 35 times. | | |
| f. Complete primer extension | 72°C | 7 min |
| g. Hold | 10°C | till removal |

*Depending on Primer

PCR reaction mixture

Components	Volume
Reaction volume (μl)	20
Template(μl)	2
Distilled water (μl)	10.5
Buffer (μl)	2
Mgcl ₂ (μl)	2
Primer Forward (μl)	1
Primer reverse(μl)	1
dNTPs (μl)	1
Taq polymerase (μl)	0.5

Concentrations of stock used DNA = 25ng/μl; Buffer (with 1.5mM Mgcl₂) =10X; dNTPs =10mM (2.5mM each); Mgcl₂ =25mM; taq polymerase and Primers: 5pM each of forward and reverse

3.2.1.8 Agarose gel electrophoresis

20μl PCR product was mixed with seven μl of 3X loading dye for the detection of desired bands. 10-12 μl of product was loaded on 3% agarose with gel 7μl of EtBr (1mg/ml) for electrophoresis (BIOBEE) with 1X TAE buffer at 100V for 60-90 min. After completion of electrophoresis the gel was viewed under UV Trans-illuminator (BIOBEE). Then gel documentation was done using gel documentation unit.

3.2.1.9 Gel documentation

After completion of gel running, then it was documented by taking photograph (Alpha Innotech Fluorochem (FC2) and the gel images were saved for scoring and future reference.

3.2.1.10 Scoring of the generated bands

For marker scores were named 1, 2, 3 and 4 were given and 1 for lower band 2 for upper band and 3 for middle and 4 Not Amplified/other.

Table 2: List of candidate gene markers selected for Zinc (Zn) content in rice.

Sl.NO	Genes	Chr.No	Source	Forward primer	Reverse primer	Anealing temp	Exp Product size
1	OsYSL2a	2	NCBI	CCGCTCCCGAGATAGAGA	AAGGCCATCCCCCATGAT	NA	1010
2	OsYSL2b	2	NCBI	TCACTGCTAAGAGCCTGCAT	CTAGCTTCCGGGAGTGAAGTT	60	980
3	OsVIT1	NA	Girish Chandel et al 2011	AAGAGCGAGGCAGACCATTA	GGAATGGACGGTTTCCAGTA	63	980
4	OsNAAT1	2	Girish Chandel et al 2011	CATCTTCCTAACCGCTGGAG	CCTTTGGCAGAAGGATTTGA	58	700
5	OsNAC	3	Girish Chandel et al 2011	AGCGAGAAGCAAGCAAGAAG	ATGCCCTGGATATCGTCGTA	58	600
6	OsZIP1a	3	NCBI	GCTCTTGCTCGCTGCAATTC	CCAACCAGTACCCGTTCCTCC	59	883
7	OsZIP1b	3	NCBI	GAAGTGTTCGGCCACGATT	TGAGATGAATTGCAGCGGAGC	59.5	561
8	OsZIP3a	4	NCBI	ACCCATCATTGCCTCCATCT	AGAACCTGCATGGCCAAA	59	1131
9	OSZIP3bII	4	NCBI	GGGAATCTTGGTGCATTTCAGT	GATCACCTGAGATAAGCTTTGG	59	1104
10	OsZIP3b	4	NCBI	CCTGCTGAGGCTGAGTTGAA	CGAGAACAAAGTAACAGGCTGC	61.5	370
11	OsZIP4b	8	NCBI	TGAGCTCATCATCACCAACCGTC	CACCTCCACCATCAAGGACG	61.5	673
12	OsZIP8a	7	NCBI	ATGAGGACGAACACCACCAC	CGGAGGGAGGGAGTAGTAATG	67	880
13	OsZIP8b	7	NCBI	GGGAGTAGTACGTACGTTTCAAATA	GCTCCCTTTCCCTCTTTACAT	52	1064
14	OsZIP8c	7	NCBI	TGTAAAGAGGGAAAAGGGAGCTA	GGCGAGTACATTCCTTCCATT	52	927

Table 3: List of candidate gene markers selected for Iron (Fe) content in rice.

Sl. No.	Marker Name	Ch. No.	Source	Forward Primer (5'-3')	Reverse primer (5'-3')	Ann. Temp (°C)	Expected product size (bp)
1	OsYSL1a	1	NCBI	TAAACCAAAGATGGCAGACC	GAACATGCCGGTTGATGATT	56	995
2	OsYSL1b	1	NCBI	GCTCGAGGCGTACAAGGAG	ATCGACGGCGAGCTTCTG	58	984
3	OsYSL1c	1	NCBI	AGGAACCAGGTGTTCTGAG	ACCTCCTGGATCTGGCTTCT	52	926
4	OsYSL4a	5	NCBI	GCAAACACTACCCCAAAGC	CATGCGGGTCTCTGACATTA	56	999
5	OsYSL4b	5	NCBI	AGCTCTGCGGGTGAACATAC	GCACGAAAAACGGAGCAGT	63	890
6	OsYSL4c	5	NCBI	TATTAGTGAGGGCGATCCAC	CACAACCACTGCCTGAAGAA	50	934
7	OsYSL4d	5	NCBI	TCTAGAACGGTCACACAGAAAA	GTAGCTATTAATTTGGGGGACA	60	917
8	OsYSL4e	5	NCBI	TATGCATGCGGTGGATGA	GTACTTTGGGAGGCCCTTCA	55	851
9	OsYSL4f	5	NCBI	CACCTCTACATCCCGAGCAT	CAACCAAAGCTGGCTCACAT	58	523
10	OsMTP1a	5	NCBI	TCTCTCCTCCCATCTCCAA	GCTGGTTACAGCGAAAGCAC	60	961
11	OsMTP1b	5	NCBI	GGGGACCTTCTTGTTGTTGG	GCCATTCTCTCCCTTGCATC	60	934
12	OsMTP1c	5	NCBI	CGGTCGAAGTGGTTGGAG	GATCAGCGTCTGCGTCCT	64	969
13	OsMTP1d	5	NCBI	TGCCATGTGACAATCACTCA	ATTATTTCCCATGCCAGTGC	55	519
14	OsNAS3a	7	NCBI	TCACCAGTTGGAGCTAATCG	GCGATGATGACGGAGTTGAT	56	904
15	OsNAS3b	7	NCBI	CACCAGAAGATGGAGGACAT	CGCAACAGAGACAATGGTTG	63	386

IV. EXPERIMENTAL RESULTS

The present investigations dealt with diversity assessment for zinc and iron content in a set of eight rice genotypes based on phenotypic and candidate gene based markers. The experiments were conducted at the Department of Plant Biotechnology, UAS, Bangalore during 2012-13. Results of the present investigation are presented under the following subheadings.

4.1 Variability and genetic parameters estimated for growth, yield and yield components under aerobic conditions

4.1.1 Analysis of Variance

The analysis of variance was worked in eight genotypes of rice for zinc and iron content in grain, leaf, culm and roots, in addition to various yield and yield attributing traits. The mean sum of squares due to genotypes and error are presented separately in Table 5 and 6. As evident from Table 5 the traits, plant height (1345.81), grain yield per plant (43.43), straw yield per plant (102.18), root number (1104.07), root volume (566.23), days to fifty per cent flowering (618.29). Similarly, from table 6 the traits grain zinc (142.46), grain iron (7.97), leaf zinc (100.43), leaf iron (121.47), culm zinc (367.23), root zinc (458.52) and root iron (286974.09) were found significant. While characters such as panicle length, total number of tillers, number of productive tillers, root length and culm iron were found non-significant.

4.2 Genetic parameters

The variability analysis was carried out in the present investigation for eight rice accessions. The range, mean, phenotypic and genotypic variances, heritability estimates and the genetic advance as per cent of mean in respect of the characters considered for present investigation are provided in Table 6 and 7. The results obtained in respect of these variability parameters are presented below.

4.2.1 Plant height (cm)

The plant height ranged from 48.00 cm to 125.00 cm with a mean value of 95.00 cm. The genotype, Devamallige recorded a maximum height (125.00 cm) while, the genotype IR-20 recorded the least height (48.00 cm).

The genotypic co-efficient of variation of 22.09 per cent and a phenotypic co-efficient of variation of 22.70 per cent were recorded for the trait. A heritability estimate of 94.70 per cent was recorded for the trait while, the genetic advance as per cent of mean was 44.30 cm.

4.2.2 Panicle length (cm)

The panicle length values ranged from 13.00 to 29.00 cm with an average panicle length of 20.08 cm. The highest panicle length was recorded in Azucena (29.00). The lowest panicle length was registered in BI-33 (13.00).

The genotypic and phenotypic coefficients of variation for panicle length were 14.16 and 19.26 per cent respectively for the trait. The genetic advance as per cent of mean was 21.43 per cent with a heritability estimate of 54.00 per cent.

4.2.3 Total number of tillers per plant

The total number of tillers per plant values ranged from 9.00 to 20.00 with an average total number of tillers per plant of 13.25. The highest total number of tillers per plant was recorded in BI-33 (20.00). The lowest number of tillers was found in Jeerigesanna (9.00).

The genotypic and phenotypic coefficients of variation for total number of tillers per plant were 6.81 and 22.20 per cent respectively. The genetic advance as per cent of mean was 3.55 per cent with a heritability estimate of 7.80 per cent.

4.2.4 Number of productive tillers per plant

The number of productive tillers per plant values ranged from 7.00 to 18.00 with an average number of productive tillers per plant of 11.25. The highest number of productive tillers per plant was recorded in BI-33 (18.00). The lowest number of tillers was found in Black rice (7.00).

The genotypic and phenotypic coefficients of variation for number of productive tillers per plant were 9.60 and 23.08 per cent respectively for the trait. The genetic advance as per cent of mean was 8.22 per cent with a heritability estimate of 17.00 per cent.

4.2.5 Grain yield per plant (g)

The grain yield per plant values ranged from 7.50 to 18.79 g with an average yield of 11.73 g. The highest grain yield per plant was recorded in BI-33 (18.75 g). The lowest grain yield per plant was registered in Jeerigesanna (7.50 g).

The genotypic and phenotypic coefficients of variation for grain yield per plant were 39.07 and 46.80 per cent respectively for the trait. The genetic advance as per cent of mean was 67.17 per cent with a heritability estimate of 69.70 per cent.

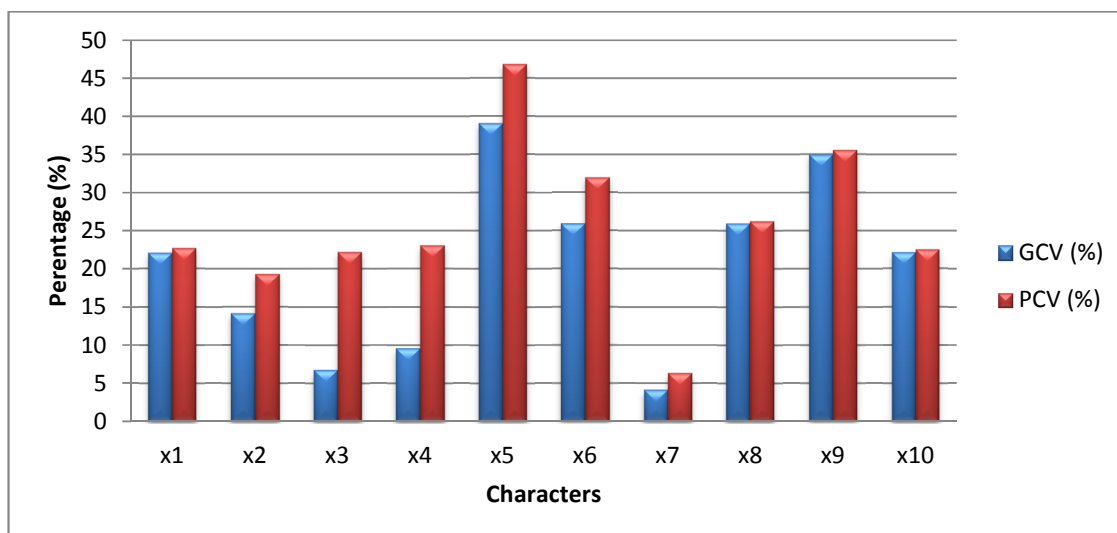


Fig: 3 Genotypic and phenotypic coefficient of variation for different phenotypic traits

X1-Plant height (cm)
 X3-Total number of tillers
 X5-Grain yield per plant
 X7-Days to fifty per cent flowering
 X9-Root volume (cm³)

X2-Panicle length (cm)
 X4-Number of productive tillers
 X6-Straw yield per plant
 X8-Root number
 X10-Root length (cm)

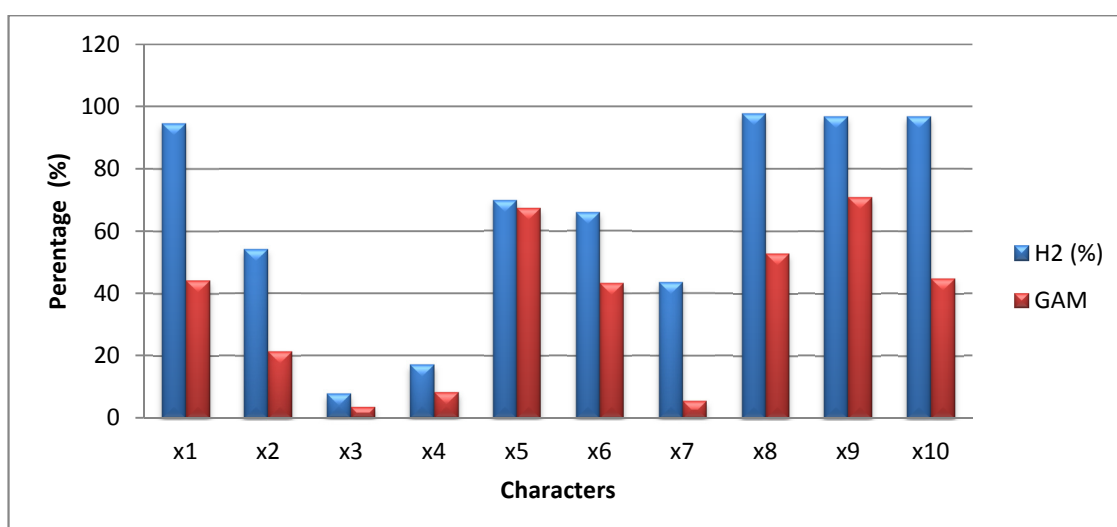


Fig: 4 Heritability (h^2 per cent) and Genetic advance as per cent of mean (GAM) for different phenotypic traits

X1-Plant height (cm)
 X3-Total number of tillers
 X5-Grain yield per plant
 X7-Days to fifty per cent flowering
 X9-Root volume (cm³)

X2-Panicle length (cm)
 X4-Number of productive tillers
 X6-Straw yield per plant
 X8-Root number
 X10-Root length (cm)

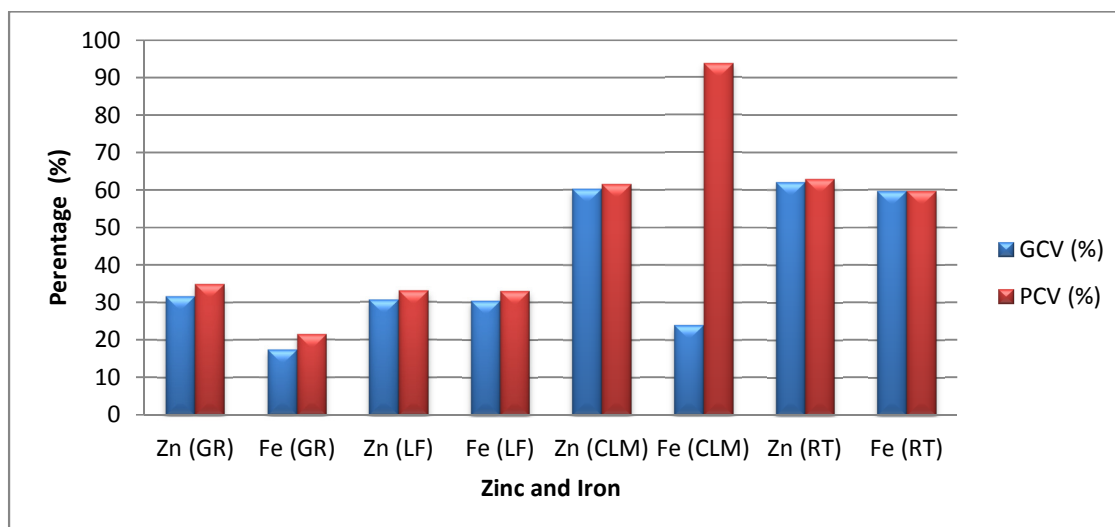


Fig: 5 Genotypic and phenotypic coefficients of variation for zinc and iron content (ppm) in grains, leaves, culm and roots

Zn (GR) =Zinc in grains Fe (GR) =Iron in grains
 Zn (LF) =Zinc in leaves Fe (LF) =Iron in leaves
 Zn (CLM) =Zinc in culm Fe (CLM) =Iron in culm
 Zn (RT) =Zinc in roots Fe (RT) =Iron in roots

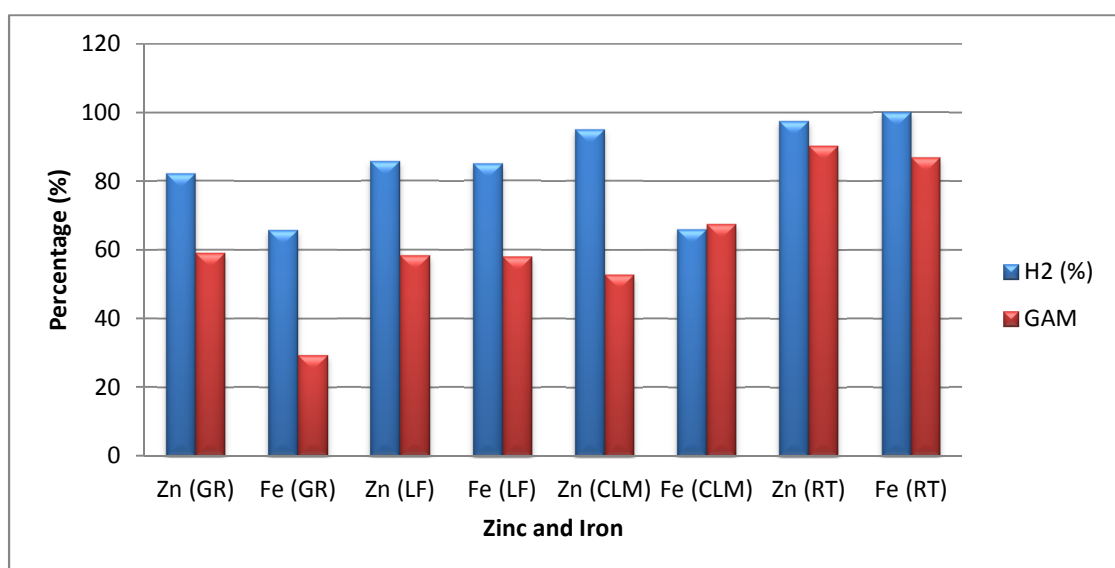


Fig: 6 Heritability (h^2 per cent) and genetic advance as per cent mean (GAM) for zinc and iron content in grains, leaves, culm and root.

Zn (GR) =Zinc in grains Fe (GR) =Iron in grains
 Zn (LF) =Zinc in leaves Fe (LF) =Iron in leaves
 Zn (CLM) =Zinc in culm Fe (CLM) =Iron in culm
 Zn (RT) =Zinc in roots Fe (RT) =Iron in roots

4.2.6 Straw yield per plant (g)

The straw yield per plant values ranged from 12.00 to 35.00 g with an average yield of 20.79 g. The highest straw yield per plant was recorded in BI-33 (35.00 g). The lowest straw yield per plant was found in Jeerigesanna (12.00 g).

The genotypic and phenotypic coefficients of variation for straw yield per plant were 25.92 and 31.94 per cent respectively. The genetic advance as per cent of mean was 43.34 per cent with a heritability estimate of 65.90 per cent.

4.2.7 Days to fifty per cent flowering

The days to fifty per cent flowering ranged from 95.00 to 119.00 days with mean value of 102.71 days. The highest and lowest days to fifty per cent flowering were recorded for the accessions Azucena (119.00) and Jeerigesanna (95.00) days respectively.

The days to fifty per cent flowering had a genotypic and phenotypic coefficient of variation of 4.19 and 6.35 respectively. The heritability estimated was 43.50 per cent with genetic advance of 5.68 per cent for the trait considered.

4.2.8 Root number

The root number values ranged from 52.00 to 119.00 with an average root number of 73.58. The highest root number was recorded in Black rice (119.00). The lowest root number was found in BJ-21 (52.00).

The genotypic and phenotypic coefficients of variation for root number were 25.97 and 26.26 per cent respectively. The genetic advance as per cent of mean was 52.92 per cent with a heritability estimate of 97.80 per cent.

4.2.9 Root volume (cm³)

The root volume values ranged from 20.00 to 61.00 cm³ with an average root volume of 39.04 cm³. The highest root volume was recorded in Black rice (61.00 cm³). The lowest root volume was recorded in IR-20 (20.00 cm³).

The genotypic and phenotypic coefficients of variation for root volume were 35.00 and 35.57 per cent respectively for a trait. The genetic advance as per cent of mean was 70.95 per cent with a heritability estimate of 96.80 per cent.

4.3.0 Root length (cm)

The root length values ranged from 41.00 to 91.00 cm with an average root length of 64.50 cm. The highest root length was recorded in Azucena (91.00 cm). The lowest root length was found in IR-20 (41.00 cm).

The genotypic and phenotypic coefficients of variation for root length were 22.14 and 22.50 per cent respectively for a trait. The genetic advance as per cent of mean was 44.86 per cent with a heritability estimate of 96.80 per cent.

4.3.1 Grain zinc content

The grain zinc content values ranged from 7.83 to 28.57 ppm with an average zinc content of 17.08 ppm. The highest grain zinc content was recorded in AM-65(28.57 ppm). The lowest grain zinc content was recorded in Azucena (7.83 ppm).

The genotypic and phenotypic coefficients of variation for grain zinc content were 31.66 and 34.91 per cent respectively. The genetic advance as per cent of mean was 59.14 per cent with a heritability estimate of 82.20 per cent.

4.3.2 Grain iron content

The grain iron content values ranged from 6.18 to 10.47 ppm with an average iron content of 8.65 ppm. The highest grain iron content was recorded in Devamallige (10.47ppm). The lowest grain iron content was recorded in Azucena (6.18 ppm).

The genotypic and phenotypic coefficients of variation for grain iron content were 17.39 and 21.45 per cent respectively. The genetic advance as per cent of mean was 29.04 per cent with a heritability estimate of 65.70 per cent.

4.3.3 Leaf zinc content

The leaf zinc content values ranged from 12.47 to 24.07 ppm with an average zinc content of 18.39 ppm. The highest leaf zinc content was recorded in Devamallige (24.07 ppm). The lowest leaf zinc content was recorded in AM-65 (12.47 ppm).

The genotypic and phenotypic coefficients of variation for leaf zinc content were 30.62 and 33.07 per cent respectively. The genetic advance as per cent of mean was 58.41 per cent with a heritability estimate of 85.70 per cent.

4.3.4 Leaf iron content

The leaf iron content values ranged from 13.56 to 30.24 ppm with an average leaf iron content of 20.30 ppm. The highest leaf iron content was recorded in Black rice (30.24 ppm). The lowest leaf iron content was recorded in BI-33 (13.56 ppm).

The genotypic and phenotypic coefficients of variation for leaf iron content were 30.49 and 33.00 per cent respectively. The genetic advance as per cent of mean was 58.04 per cent with a heritability estimate of 85.00 per cent.

4.3.5 Culm zinc content

The culm zinc content values ranged from 4.93 to 33.37 ppm with an average culm zinc content of 18.22 ppm. The highest culm zinc content was recorded in Azucena (33.37 ppm). The lowest culm zinc content was recorded in AM-65 (4.93 ppm).

The genotypic and phenotypic coefficients of variation for culm zinc content were 60.28 and 61.57 per cent respectively. The genetic advance as per cent of mean was 52.87 per cent with a heritability estimate of 95.00 per cent.

4.3.6 Culm iron content

The culm iron content values ranged from 19.14 to 71.61 ppm with an average culm iron content of 34.07 ppm. The highest culm iron content was recorded in Devamalligae (71.61 ppm). The lowest culm iron content was recorded in AM-65 (19.14 ppm).

The genotypic and phenotypic coefficients of variation for culm iron content were 24.03 and 93.67 per cent respectively. The genetic advance as per cent of mean was 67.54 per cent with a heritability estimate of 66.00 per cent.

4.3.7 Root zinc content

The root zinc content values ranged from 7.10 to 37.73 ppm with an average root zinc content of 19.81 ppm. The highest root zinc content was recorded in BJ-21 (37.73 ppm). The lowest root zinc content was recorded in Azucena (7.10 ppm).

The genotypic and phenotypic coefficients of variation for root zinc content were 62.12 and 62.95 per cent respectively. The genetic advance as per cent of mean was 90.26 per cent with a heritability estimate of 97.40 per cent.

4.3.8 Root iron content

The root iron content values ranged from 261.77 to 854.66 ppm with an average root iron content of 518.52 ppm. The highest root iron content was recorded in AM-65 (854.66 ppm). The lowest root iron content was recorded in Devamallige (261.77 ppm).

The genotypic and phenotypic coefficients of variation for root iron content were 59.65 and 59.65 per cent respectively. The genetic advance as per cent of mean was 86.87 per cent with a heritability estimate of 100.00 per cent.

Table 4: Analysis of variance for yield and its associated traits in selected rice genotypes

Mean sum of square											
Source of variation	Df	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10
Genotype	7	1345.81*	31.12	9.50	8.61	43.43*	102.18*	79.47	1104.07*	566.23*	618.29*
Replication	2	11.38	0.04	2.04	2.54	16.84	6.54	23.29	1.54	10.29	2.38
Error	14	26.33	7.85	8.38	5.68	6.45	16.26	24.15	9.16	5.48	7.38
SEM		2.96	1.62	1.67	1.38	1.46	2.33	2.84	1.75	1.35	1.57
CD @ 5 per cent		8.99	4.91	5.07	4.18	4.43	7.06	8.61	5.30	4.10	4.76
CV @ 5 per cent		5.40	13.95	22.40	21.75	21.58	19.39	4.78	4.11	5.99	4.20

* Significant at 5 per cent

X1-Plant height (cm)

X3-Total number of tillers per plant

X5-Grain yield per plant (g)

X7-Root length (cm)

X9-Root volume (cm³)

X2-Panicle length (cm)

X4-Number of productive tillers

X6-Straw yield per plant

X8-Root number

X10-Days to fifty per cent flowering



Plate: 3 Phenotyping of roots at maturity

Table 5: Analysis of variance for zinc and iron (ppm) content in grains, leaves, culm and roots of selected rice genotypes

Means sums of square									
Source of variation	df	Zn (GR)	Fe (GR)	Zn (LF)	Fe (LF)	Zn (CLM)	Fe (CLM)	Zn (RT)	Fe (RT)
Genotype	7	142.46*	7.97*	100.43*	121.47*	367.23*	884.44	458.52*	286974.09*
Replication	2	0.35	1.22	4.87	6.41	8.37	1078.40	7.34	1.95
Error	14	5.05	1.17	5.34	6.58	4.79	1086.68	3.64	9.59
SEM		1.29	0.63	1.33	1.48	1.26	19.03	1.10	1.79
CD @ 5 per cent		3.935	1.90	4.05	4.49	3.83	57.73	3.34	5.42
CV @ 5 per cent		13.160	12.53	12.56	12.64	12.01	96.75	9.63	0.60

* Significant at 5 per cent

Zn (GR) =Zinc in grains

Zn (LF) =Zinc in leaves

Zn (CLM) =Zinc in culm

Zn (RT) =Zinc in roots

Fe (GR) =Iron in grains

Fe (LF) =Iron in leaves

Fe (CLM) =Iron in culm

Fe (RT) =Iron in roots

Table 6: Genetic variability parameters for different phenotypic traits among selected rice genotypes.

Character	Min	Max	Mean	GCV	PCV	h ² per cent	GAM
X1	48.00	125.00	95.00	22.09	22.70	94.70	44.30
X2	13.00	29.00	20.08	14.16	19.26	54.00	21.43
X3	9.00	20.00	13.25	6.81	22.20	7.80	3.55
X4	7.00	18.00	11.25	9.60	23.08	17.00	8.22
X5	7.5	18.79	11.73	39.07	46.80	69.70	67.17
X6	12.00	35.00	20.79	25.92	31.94	65.90	43.34
X7	95.00	119.00	102.71	4.19	6.35	43.50	5.68
X8	52.00	119.00	73.58	25.97	26.26	97.80	52.92
X9	20.00	61.00	39.04	35.00	35.57	96.80	70.95
X10	41.00	91.00	64.50	22.14	22.50	96.80	44.86

X1-Plant height (cm)

X3-Total number of tillers

X5-Grain yield per plant (gm)

X7-Days to fifty per cent flowering

X9-Root volume (cm³)

Min = Minimum

GCV = Genotypic coefficient of variation

PCV = Phenotypic coefficient of variation

X2-Panicle length (cm)

X4-Number of productive tillers

X6-Straw yield per plant

X8-Root number

X10-Root length (cm)

Max = Maximum

h² per cent = heritability per cent

GAM = Genetic advance as per cent mean

Table 7: Genetic variability parameters for different quantitative traits among selected rice genotypes for zinc and iron content (ppm) in grains, leaves, culm and roots.

Character (ppm)	Min	Max	Mean	GCV	PCV	h² per cent	GAM
Zn (GR)	7.83	28.57	17.08	31.66	34.91	82.20	59.14
Fe (GR)	6.18	10.47	8.65	17.39	21.45	65.70	29.04
Zn (LF)	12.47	24.07	18.39	30.62	33.07	85.70	58.41
FE (LF)	13.56	30.24	20.30	30.49	33.00	85.00	58.04
Zn (CLM)	4.93	33.37	18.22	60.28	61.57	95.00	52.87
Fe (CLM)	19.14	71.61	34.07	24.03	93.67	66.00	67.54
Zn (RT)	7.1	37.73	19.81	62.12	62.95	97.40	90.26
Fe (RT)	61.76	854.66	518.52	59.65	59.65	100.00	86.87

GCV=Genotypic Coefficient of Variation
h² per cent=Heritability per cent in Broad sense

PCV=Phenotypic Coefficient of variation
GAM=Genetic Advance as Per cent Mean

4.3 Correlation analysis

The selection for specific character was known to result in correlated response in certain other character (Shivkumar, 2011). The improvement on seed yield per plot, the most important target of the character in most of the crops, can be achieved by direct selection through other easily observable characters. But, this needs a good understanding of association of different components with yield and their possible associations among themselves. The genetic and phenotypic correlation was determined to know the nature of relationship existing between the eight rice genotypes for 12 quantitative characters. The inter character correlations at genotypic and phenotypic levels among 12 productivity traits under the present investigation were worked out which are presented in Table 8.

4.3.1 Plant height

The plant height exhibited negative and significant association with number of productive tillers (-0.72). The plant height showed negative non-significant association with grain yield per plant (-0.61), grain zinc content (-0.17) and grain iron content (0.04)

4.3.2 Total number of tillers per plant

The total number of tillers per plant exhibited positive and significant association with number of productive tillers per plant (0.91) and grain yield per plant (0.845) both at the genotypic level and phenotypic level. The total number of tillers exhibited negative non-significant association with days to fifty per cent flowering (-0.2), root number (-0.09) root length (-0.03) and grain iron content (-0.58)

4.3.3 Number of productive tillers

The number of productive tillers showed a positive and significant association with grain yield per plant (0.87). The number of productive tillers exhibited negative non-significant association with days to fifty per cent flowering (-0.5), root number (-0.43), root volume (-0.16), root length (-0.29), grain zinc (-0.10) and grain iron content (-0.39).

4.3.4 Grain yield per plant

Grain yield per plant showed negative non-significant association with grain zinc content (-0.05) and grain iron content (-0.40).

4.3.5 Straw yield per plant

Straw yield per plant exhibited negative significant correlation with grain iron content (-0.73) and non-significant association with grain zinc content (0.26) and grain yield per plant.

Table 8: Estimates of phenotypic correlation coefficients for 12 characters in selected rice genotypes

Characters	PHT	PNL	TNOT	NPT	G.YLD / PT	S. YLD / PT	DFF	RT NOR	RT VOL	RT LT	ZN (GR)	FE (GR)
PHT	1	0.546	-0.6	-.724*	-0.616	0.191	0.47	0.211	0.294	0.624	-0.179	0.045
PNL		1	-0.6	-0.4	-0.147	-0.283	0.23	-0.045	-0.249	0.338	-0.582	0.182
NOT			1	.915**	.845**	0.472	-0.2	-0.097	0.188	-0.037	0.051	-0.583
NPT				1	.872**	0.325	-0.5	-0.438	-0.162	-0.296	-0.106	-0.393
G.YLD/PT					1	0.322	-0.2	-0.138	-0.048	-0.009	-0.058	-0.402
S.YLD/PT						1	0.46	0.024	0.554	0.624	0.206	-.731*
DFF							1	0.556	0.624	.864**	0.251	-0.493
RT NOR								1	.715*	0.482	0.528	-0.016
RT VOL									1	0.66	0.311	-0.528
RT LT										1	0.008	-0.599
ZN (GR)											1	0.172
FE (GR)												1

* Significance at 5 per cent

** Significance at 1 per cent

PHT=Plant height (cm)

NPT=Number of productive tillers

DFF=Days to 50 per cent flowering

RT NOR=Root number

RT LT=Root length

FE (GR) =Iron grains

PNL=Panicle length

G.YLD/PLT=Grain yield per plant

S.YLD/PLT =Straw yield per plant

RT VOL=Root volume

ZN (GR) =Zinc grains

TNOT= Total number of tillers

Table 9: Micronutrient content iron and zinc content (ppm) in different plant tissues

Genotype	Grain yield per plant (g)	Zn (GR)	Fe (GR)	Zn (LF)	Fe (LF)	Zn (CLM)	Fe (CLM)	Zn (RT)	Fe (RT)
BI-33	18.79	14.43	6.72	15.42	13.48	5.11	41.19	22.85	281.47
Azucena	11.47	7.83	6.18	12.47	17.09	33.37	28.92	7.10	500.93
Jeerigesanna	7.58	10.87	9.90	14.07	13.56	31.93	23.45	3.97	250.97
Devamallige	10.8	20.07	10.47	24.07	28.77	24.50	31.61	11.39	261.77
IR-20	16.21	19.00	9.37	20.89	19.58	19.43	20.69	26.47	945.10
Black rice	10.23	21.2.	9.67	28.42	30.24	13.75	28.19	33.66	541.33
AM-65	9.47	28.57	7.39	12.72	17.62	4.93	19.15	15.33	854.67
BJ -21	9.30	12.67	9.50	19.09	22.02	12.76	39.37	29.73	711.93

Zn (GR) = Zinc in grains
 Zn (LF) = Zinc in leaves
 Zn (CLM) = Zinc in culm
 Zn (RT) = Zinc in roots

Fe (GR) = Iron in grains
 Fe (LF) = Iron in leaves
 Fe (CLM) = Iron in culm
 Fe (RT) = Iron in roots

4.3.6 Days to fifty per cent flowering

Days to fifty per cent flowering exhibited positive and significant association with root length (0.86). However days to fifty per cent flowering showed non-significant association with grain zinc and iron content.

4.3.7 Root number

The root number showed a positive and significant association with root volume (0.71) at genotypic level. The root number also exhibited negative non-significant association with grain zinc and grain iron content.

Among the selected genotypes zinc and iron content was estimated in different tissues of rice plant like grain, leaves, culm and root. Highest grain zinc content was recorded by AM-65 (28.57 ppm), while grain iron content was recorded by Devamallige (10.47 ppm). Black rice has recorded the highest leaf zinc (28.42 ppm) and leaf iron (30.24 ppm) content. Genotypes BI-33 and IR-20 has shown highest grain yield, while these genotypes has recorded the lowest grain iron (6.72 ppm and 9.37 ppm) and grain zinc content (14.43 ppm and 19.0 ppm) respectively (Table 9).

The genotype Azucena has recorded the highest for culm zinc (33.37 ppm) content and culm iron (41.19 ppm) content was recorded in case of BI-33. Highest root zinc recorded by Black rice (33.66 ppm), while the root iron content was recorded highest in case of IR-20 (945.10 ppm).

4.4 Relationship among grain zinc, grain iron and yield per plant for the selected genotypes during *Kharif*-2013.

4.4.1 Relationship between grain zinc and iron (ppm) content and grain yield per plant (g)

Assessment of the relationship between grain zinc and grain yield per plant among the selected genotypes using linear regression showed that (Fig.7) there is a linear relationship, indicating that unit increase in grain yield per plant resulted in decreased grain iron content. In (Fig.8) clearly shows linear regression between grain iron content and grain yield per plant, indicating that increasing grain iron content resulted in decreased grain yield per plant.

Relationship between grain zinc content and grain iron content was linear in relationship indicating that unit increase in the grain zinc content resulted in increase in the grain iron content (Fig 9).

4.5 Molecular analysis of rice genotypes using candidate gene markers for zinc and iron content in rice grains.

Use of DNA- based markers in indirect selection for traits in molecular genetics is known as marker assisted selection (MAS). Along with complete phenotypic characterization of all eight genotypes, genotypic characterization was also performed with the help of trait specific candidate gene markers. Total genomic DNA was extracted by using CTAB method and PCR reaction was performed and PCR product was run on agarose gel 3 per cent for its profiling. All 29 markers used are already reported in literature along with their associated traits. Amplified product size was compared with already reported band size in NCBI database and GRAMENE database.

4.5.1 Screening with candidate gene markers

Molecular characterization was carried out using 29 candidate gene specific primers for zinc and iron transporters. All primers showed monomorphic banding pattern indicated absences of Indels in the targeted gene loci.

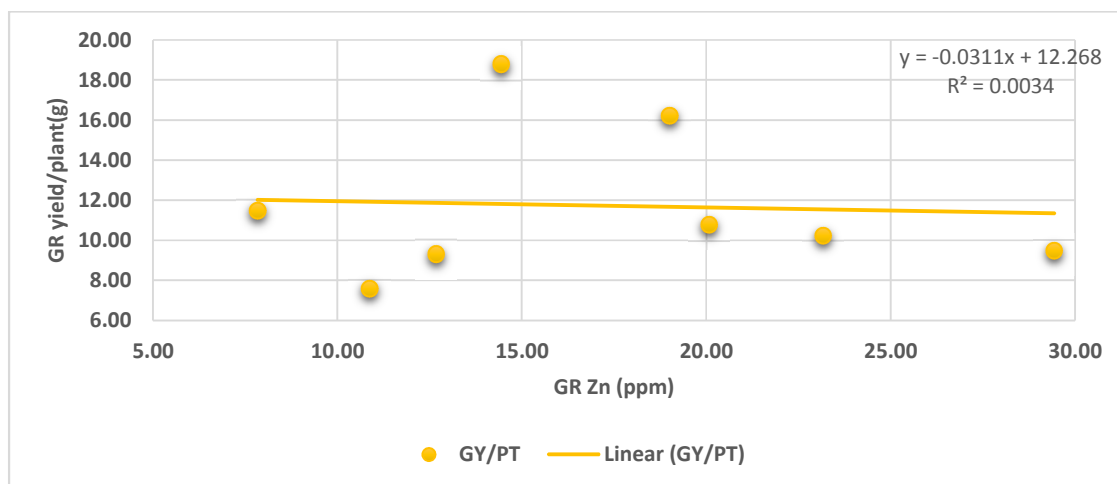


Fig: 7 Relationship between grain zinc content (ppm) and grain yield per plant (g)

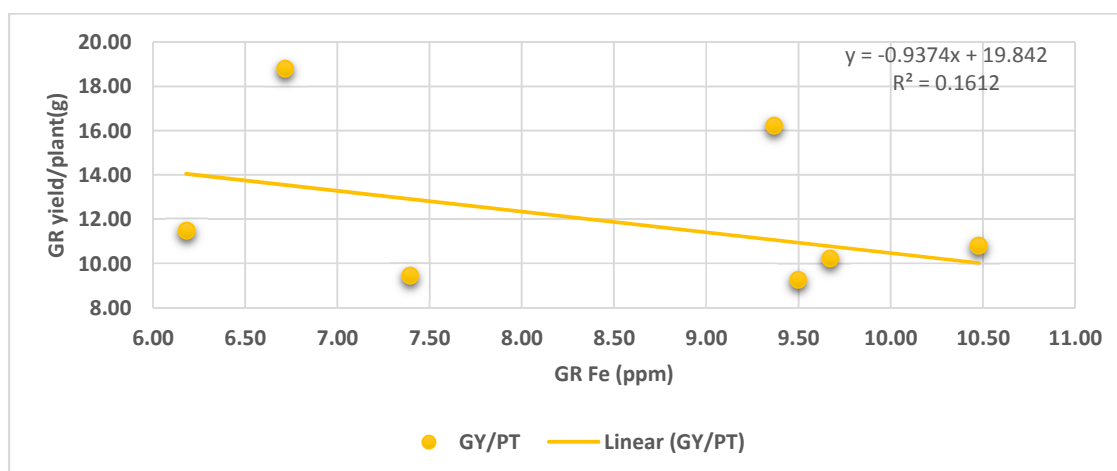


Fig: 8 Relationship between grain iron content (ppm) and grain yield per plant (g)

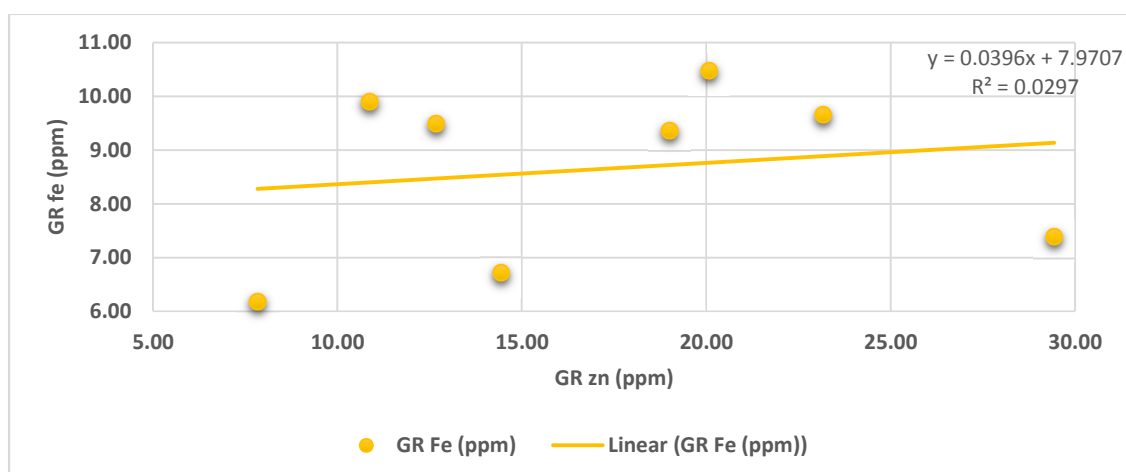


Fig: 9 Relationship between grain iron and grain zinc content (ppm)

V. DISCUSSION

Rice is a staple food for more than 50 per cent of the population around the world, which is a poor source of iron and zinc. Zinc and iron accumulation in grains of rice involves a polygenic inheritance which is attributed to the interactions of a number of genes located on different regions of the chromosomes (Avendano, 2000; Lu *et al.*, 2008; Garcia-Olivera *et al.*, 2009; Zhang *et al.*, 2011).

The availability of rice genome sequence has given opportunity for identifying candidate genes involved in zinc accumulation in grains. Identifying QTLs/genes for zinc in rice grains can help in biofortification programs. Molecular markers tightly linked to the genes (QTLs) responsible for high zinc content can be used for devising the plant breeding strategies for improving grain micronutrient content through MAS. Thus, to facilitate this approach preliminary steps are required to characterize molecular markers linked to QTLs for grain zinc content and establish their association with phenotypic variation and also the effect of environment on the trait. The results obtained from this study on various aspects are discussed below.

5.1 Phenotypic studies among selected rice genotypes for zinc and iron in grains, leaves, culm and root and other yield related characters

5.1.1 Analysis of variance

In the present study, eight rice genotypes were evaluated for zinc and iron content of grain, leaves, culm and root and other yield related traits during *Kharif*-2013. Analysis of variance revealed highly significant variation in plant height, grain yield per plant, straw yield per plant, root volume, root number, and days to 50 per cent flowering, grain zinc and iron content, leaf zinc and iron content, root zinc and iron content as shown in (Table 4 and 5).

5.1.2 Mean performance of selected rice genotypes

The mean performances of different quantitative traits were studied in selected rice genotypes grown in *Kharif*-2013 are shown in Tables 6 and 7.

Considerable amount of genetic variability was observed for yield related traits and grain zinc content in selected genotypes, which can be exploited for crop improvement. The range and mean values reflect the extent of variability among the genotypes. The wide range for different quantitative traits among genotypes could be due to their variability in adaptation to aerobic growth conditions.

5.1.3 Genetic variability parameters

The trait improvement in a plant depends on the existence of the genetic variability and heritability. Prior knowledge on the estimates of variability in the

population and heritable components of the trait are useful tools in any breeding programme. Thus, information on both phenotypic and genotypic coefficient of variation and heritability of the character would be advantageous to breeding.

Hence, the co-efficients of variation expressed at phenotypic and genotypic levels have been used to compare the variability observed among different characters. While, genotypic co-efficient of variation indicates the amount of genetic variability present in the character, the heritability estimates aid in determining the relative amount of heritable portion of variation. However, heritability values themselves provide no indication of the amount of genetic progress that would result from selecting the best individuals. Heritability estimates in broad sense would be reliable when accompanied by high genetic advance (Burton and De Vane, 1953, Johnson *et al.*, 1955). The broad sense heritability is the relative magnitude of genotypic to phenotypic variances for the traits and it is used as a predictive tool in selection procedures (Allard, 1960).

In this study, the phenotypic coefficients of variation (PCV) and genotypic coefficients of variation (GCV) were highest for plant height, grain yield per plant, straw yield, root number, root volume and root length. These observations suggest that, these traits were relatively greater under the influence of genetic factors and relatively less influence of the environment. Similar results have been reported by Bisne *et al.* (2009); Akinwale *et al.*, (2011) and Gangashetty *et al.*, (2013) for grain yield per plant. High GCV and PCV was recorded for plant height and grain yield. Similar results were reported by Seyoum *et al.*, (2012), Selvaraj *et al.*, (2011) and Akinwale *et al.*, (2011).

In this study, the phenotypic coefficients of variation (PCV) and genotypic coefficients of variation (GCV) were highest for zinc content of grains, and iron content of leaves, culm and roots, except for iron content in grains, which showed low GCV and PCV. High heritability was estimated for grain zinc content by Gangashetty *et al.*, (2013).

Moderate PCV and GCV were observed for panicle length. These values indicated considerable amount of variability for these characters reported by (Gangashetty *et al.*, 2013).

Low PCV and GCV were observed for number of tillers, number of productive tillers and days to 50 per cent flowering. These coefficient values indicated lower amount of variability for this trait. Similar results were reported by Gangashetty *et al.*, (2013) and Sinha *et al.*, (2004)

High heritability estimates were recorded for grain yield per plant, and grain zinc content. Similar results were reported for grain yield per plant by Akinwale *et al.*, (2011) and Gangashetty *et al.*, (2013). High heritability estimates were recorded for grain yield per plant and plant height by Bisne *et al.*, (2009)

High heritability coupled with high genetic advance as percent mean was recorded for plant biomass, grain yield per plant, grain zinc content and harvest index. This indicated that, these traits were not much influenced by environmental factors.

Hence, these traits were mostly controlled by additive or additive X additive gene interactions are expected to respond to direct selection for trait improvement. Similar results were reported for grain yield per plant by (Suman *et al.*, 2005); Akinwale *et al.*, 2011) and Mohan *et al.*, 2012) for grain zinc content (Govindaraj *et al.*, 2011) and Ravindra babu *et al.*, (2012).

The present investigation revealed high heritability coupled with high genetic advance as percent of mean for most of the characters, indicating the presence of considerable variation and additive gene effects. Hence, improvement of these characters could be effective through phenotypic selection.

5.1.4 Correlation studies among growth, yield and micronutrient content components under aerobic condition

Correlation coefficient analysis helps to determine the nature and degree of relationship between any two measurable characters. The study of relationship of different traits has great importance in indirect selection of a complex trait associated with a simple trait. In the present study, correlation studies were done for selected rice genotypes grown in *Kharif*-2013.

In the present experiment a significant and positive correlation has been observed between total numbers of tillers with number of productive tillers. Similar results were reported by Ravindra (1996), Raju *et al.*, (2004), Akinwale *et al.*, (2011) and Nagesh *et al.*, (2012).

Number of productive tillers showed highly significant positive correlation with grain yield per plant, similar results were reported by Nagesh *et al.*, (2012) and Berhanu (2012).

Days to 50 per cent flowering showed highly significant positive correlation with root length. Similar results were reported by (Ground nut) by Thakur *et al.*, (2012). Root number showed highly significant positive correlation with root volume. Similar results were reported by Sandhu *et al.*, (2013)

5.1.5 Rice genotypes with high zinc and iron content in the grains, leaves, culm and roots

Among the selected genotypes zinc and iron content was estimated in different parts of rice plant like grains, leaves, culm and roots. Highest grain zinc content was recorded in AM-65 (28.57 ppm), while grain iron content was recorded in Devamallige (10.47 ppm). Black rice has recorded the highest leaf zinc (28.42 ppm) and leaf iron (30.24 ppm) content. Genotypes BI-33 and IR-20 has showed highest grain yield, while these genotypes has recorded the lowest grain iron (6.72 ppm and 9.37 ppm) and grain zinc content (14.43 ppm and 19.0 ppm) respectively (Table 9).

The genotype Azucena has recorded the highest for culm zinc (33.37 ppm) content and culm iron (41.19 ppm) content was recorded in case of BI-33. Highest root zinc recorded by Black rice (33.66 ppm), while the root iron content was recorded highest in case of IR-20 (945.10 ppm). Hence breeding for high grain zinc and iron content with good yielding character will be the beneficial in the biofortification.

5.1.6 Molecular evaluation of candidate gene primers in selected rice genotypes for zinc and iron content in grains grown under aerobic condition

Grain yield is a complex trait controlled by many genes, environmentally influenced and determined by the magnitude and nature of their genetic variability (Singh *et al.*, 2000). Understanding the genetic nature and inheritance of such complex trait and extent of its association with grain yield and yield related traits could help in plant breeding efforts.

Currently, the available molecular markers have facilitated genetic mapping of the QTL for accumulation of micronutrient in different cereal crop plants (Zimmerman and Hurrel, 2002; Lu *et al.*, 2008).

Molecular characterization was carried out using 29 candidate gene specific primers for zinc and iron transporters. All primers showed monomorphic banding pattern indicated absences of INDELS in the targeted gene loci. Further sequencing of these fragments may reveal SNPs for functional association.

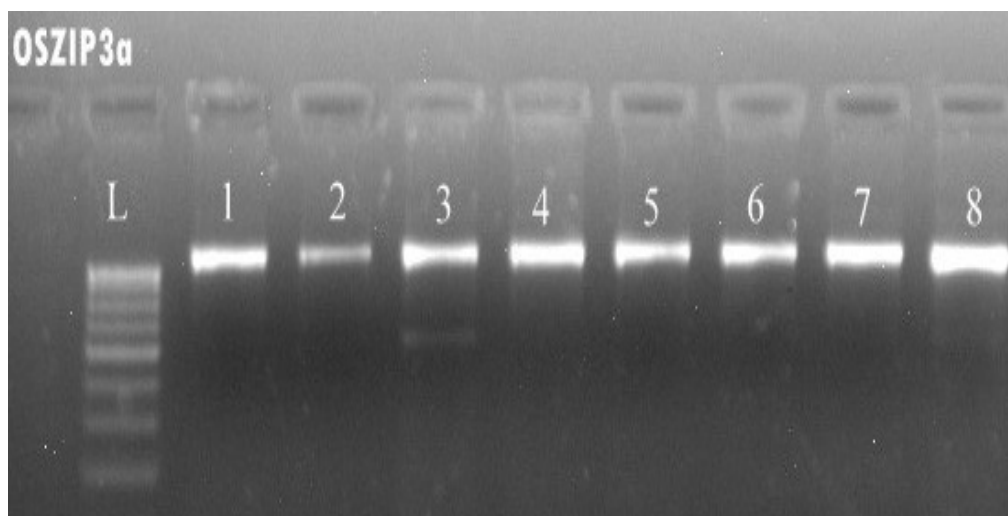


Plate: 4 Agarose gel profile of zinc candidate gene marker of OsZIP3a

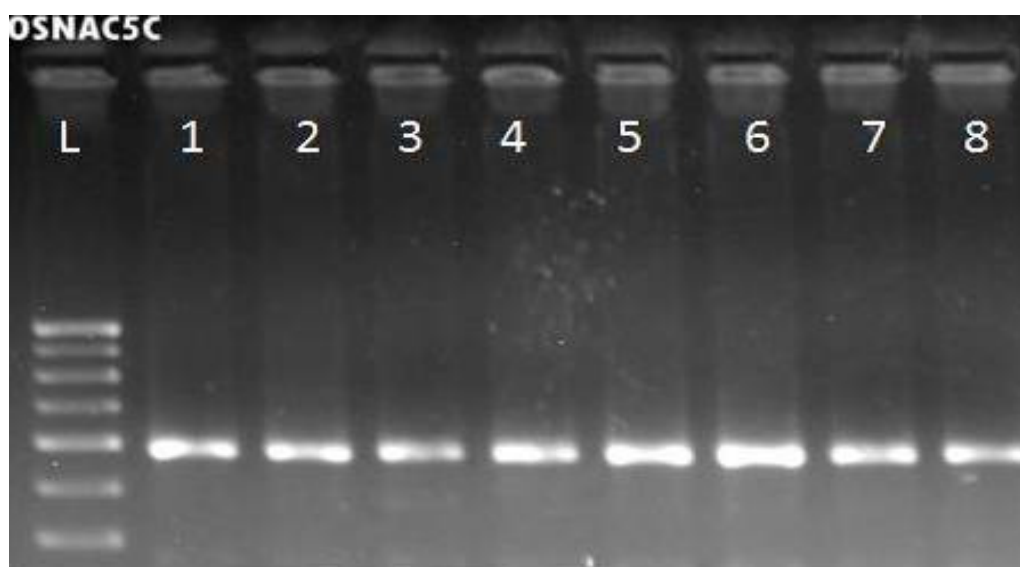


Plate: 5 Agarose gel profile of zinc candidate gene marker of OsNAC5c

L- 100 bp Ladder

1.BI-33

2.Azucena

3.Jeerigesanna

4.Devamallige

5.IR-20

6.Black rice

7.AM-65

8.BJ-21

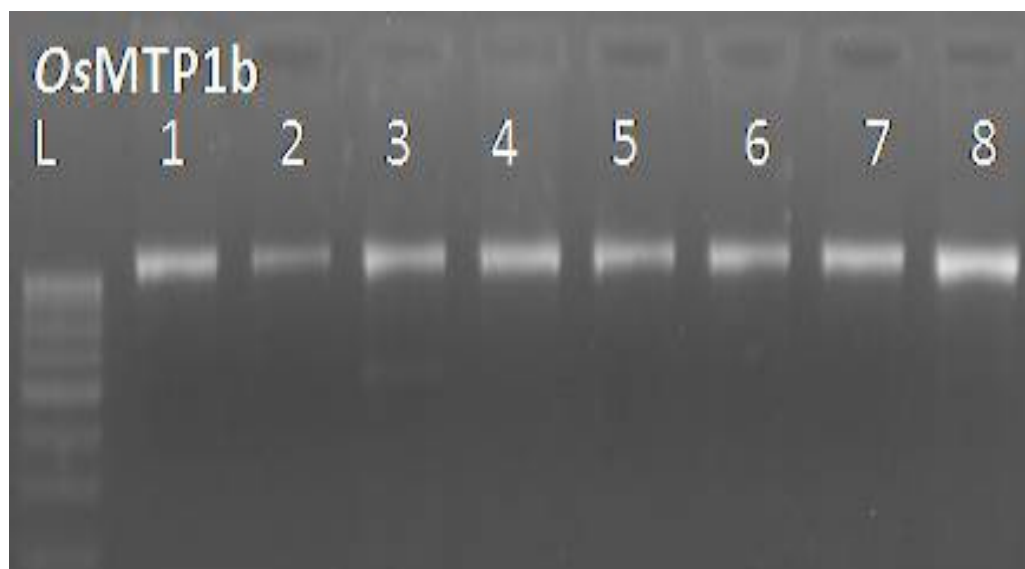


Plate: 6 Agarose gel profile of iron candidate gene marker of *OsMTP1b*

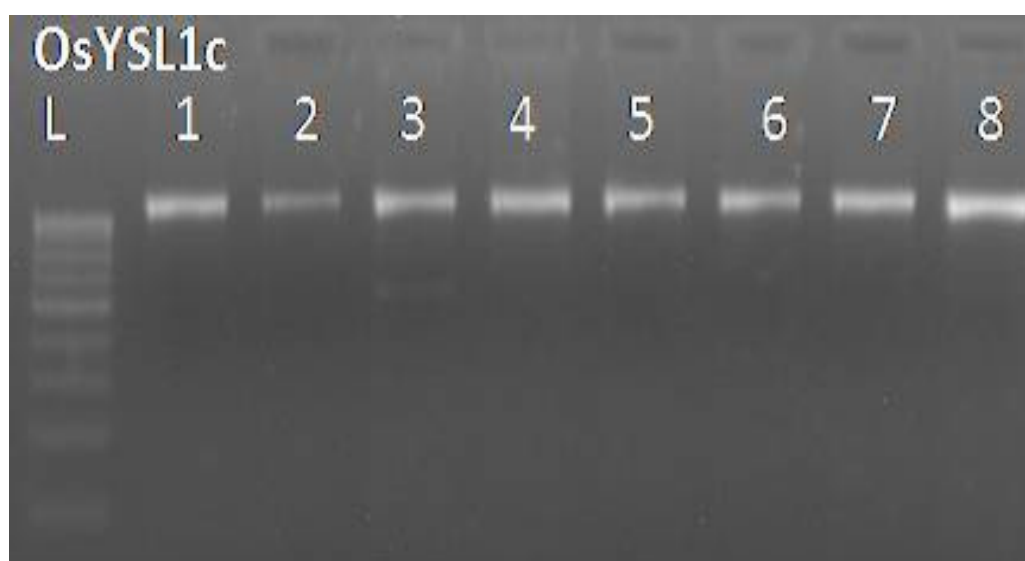


Plate: 7 Agarose gel profile of iron candidate gene marker of *OsYSL1c*

L- 100 bp Ladder

1.BI-33

2.Azucena

3.Jeerigesanna

4.Devamallige

5.IR-20

6.Black rice

7.AM-65

8.BJ-21

VI. SUMMARY

Rice is an important cereal food crop feeding more than one half of the world's population. Over 90 % of rice is produced and consumed in Asia. It accounts for more than 65% of caloric intake providing 23 % of global human per capita energy and 16 % of per capita protein.

Micronutrient deficiency is a global health problem contributing to high rate of children and women's mortality. It is estimated that more than 3.5 billion people in the world are deficient in vitamin A, iodine, iron or zinc.

In the present study eight rice genotypes grown in *Kharif*-2013 at maturity were evaluated for zinc and iron content in different tissues of plant like grain, leaf, culm and root. Analysis of variance revealed highly significant variation for plant height, grain yield per plant, straw yield, root length, root volume, days to 50 per cent flowering and zinc and iron content of grains, leaves, culm and roots.

In this study, the phenotypic coefficients of variation (PCV) and genotypic coefficients of variation (GCV) were highest for plant height, grain yield per plant, straw yield, root number, root volume and root length. These observations suggest that, these traits were under relatively high influence of genetic factors and relatively less influence of the environment. Thus, improvement of these characters could be effectively done through phenotypic selection.

High heritability coupled with high genetic advance as percent mean was recorded for plant height, grain yield per plant, straw yield, root number, root volume and root length, zinc and iron content of grains culms and roots. Hence, these traits were mostly controlled by additive and or additive X additive gene interactions and expected to respond to direct selection for trait improvement.

Variations among genotypes studied were highly significant and positive correlations were observed for total number of tillers with number productive tillers. Root number showed highly significant positive correlation with root volume. The high correlations existing between different plant traits can be used to conduct indirect selection of a desired trait in a breeding program. On the other hand grain iron content showed highly significant negative correlation with straw yield. Hence, breeding for high grain zinc and iron content and enhancement of grain yield per plant has to be designed separately.

Among the selected genotypes zinc and iron content was estimated in different tissues of rice plant like grain, leaves, culm and root. Highest grain zinc content was recorded by AM-65 (28.57 ppm), while grain iron content was recorded by Devamallige (10.47 ppm). Black rice has recorded the highest leaf zinc (28.42 ppm) and leaf iron (30.24 ppm) content. Genotypes BI-33 (18.79 g) and IR-20 (16.21g) has shown highest

grain yield, while these genotypes has recorded the lowest grain iron (6.72 ppm and 9.37 ppm) and grain zinc content (14.43 ppm and 19.0 ppm) respectively.

The genotype AM-65 showed stable for higher grain zinc (28.57 ppm) but low in grain iron content (7.39 ppm) Breeding of this genotype for better grain yield with high grain iron content will help in improvement of micronutrient content.

The genotype BI-33 is high yielding (18.79 g) with good root system, moderate grain zinc and iron content. Selection of this genotype for crossing with high zinc and iron lines will help in the development of superior variety with high micronutrient content and grain yield under aerobic conditions.

For the molecular characterization, 29 candidate gene specific primers of *OsNAAT*, *OsZIP* and *OsMTP* families for zinc and iron transporters were screened. All primers shown monomorphic banding pattern at 3 per cent agarose gel, Further sequencing of these fragments may reveal SNPs for functional association.

FUTURE LINE OF WORK

- Breeding for high grain zinc content and enhancement of grain yield per plant has to be designed separately.
- Sequencing of the amplified products obtained by amplification which may be gives variation in zinc and iron.
- SNP analysis of obtained products may be useful for identification of single nucleotide variation in zinc and iron content.

VII. REFERENCES

- AKINWALE, M. G., GREGORIO, G., NWILENE, F., AKINYELE, B. O., OGUNBAYO, S. A. AND ODIYI, A. C., 2011, Heritability and correlation coefficient analysis for yield and its components in rice (*Oryza sativa* L.). *Afr. J. Plant Sci.*, **5** (3): 207 - 212.
- AKKAYA, M. S., BHAGWAT, A. A. AND CREGAN, P. B., 1992, Length polymorphisms of simple sequence repeat DNA in soybean. *Genet.*, **132**: 1131 - 1139.
- AL-JIBOURIE, H. A., MILLER, P. A. AND ROBINSON, H. F., 1958, Genotypic and environmental variances in an upland cotton cross of interspecific origin. *Agron. J.*, **50**: 636 - 637.
- ALLARD, R. W., 1960, Principles of Plant Breeding. John Wiley and Sons, Inc. New York, p. 485.
- ALLOWAY, B. J., 2004, Zinc in soils and crop nutrition. International zinc association, Brussels, Belgium *Analysis*, **10**: 459 – 472.
- AMUDHA, K., THIYAGARAJAN, K. AND SAKTHIVEL, N., 2009, Aerobic rice: a review. *Agric. Rev.*, **30**(2): 145-149.
- ANANDAN, A., RAJIV, G., ESWARAN, R. AND PRAKASH, M., 2011, Genotypic variation and relationships between quality traits and trace elements in traditional and improved rice (*Oryza sativa* L.) genotypes. *J. Food Sci.*, **76** (4): 122 - 130.
- ANURADHA K., S. AGARWAL, A. K. BATCHU, A. P. BABU, B. P. M. SWAMY, T. LONGVAH. AND SARLA, N., 2012, Evaluating rice germplasm for iron and zinc concentration in brown rice and seed dimensions. *J. Phytol.*, **4**: 19 – 25.
- AOYAMA, T., KOBAYASHI, T., TAKAHASHI, M., NAGAAKA, S., USUDA, KANAKO., KAKEI, Y., ISHIMARU, Y., NAKANISHI, H., MORI, SATOSHI. 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**: 81-692.
- ATLIN, G. N., LAZA, M., AMANTE, M. AND LAFITTE, H. R., 2004, Agronomic performance of tropical aerobic irrigated and traditional upland rice varieties in three hydrological environments at IRRI. In: *Fourth Int. Crop Sci. Congress*, Metranavila, Philippines.

- AVENDANO, B. S., 2000, Tagging high zinc content in the grain, and zinc deficiency tolerance genes in rice (*Oryza sativa* L.) using simple sequence repeats (SSR). *M. Sc. Thesis, Laguna College, Los Banos, Philippines*
- BANERJEE, S. AND CHANDEL, G., 2011, Understanding the role of metal homeostasis related candidate genes in Fe/Zn uptake, transport and redistribution in rice using semi-quantitative RT-PCR. *J. Plant Mol. Biol. Biotechnol.*, **2(1)**: 33-46.
- BANERJEE, S., SHARMA, D. J., VERULKUR, S. B. AND CHANDEL, G., 2010, Use of insilico and semiquantitative RT-PCR approaches to develop nutrient rich rice (*Oryza sativa* L). *Indian J. Biotechnol.*, **9**: 203-212.
- BASHIR, K., LSHIMARU, Y. AND NISHIZAWA., 2010, Iron uptake and loading into the rice grains. *Rice.*, **3**: 122-130.
- BASHIR,K.,NOZOYE,T.,ISHIMARU,Y.,NAKANISHI,H.,NISHIZAWA,K.N., 2013, Exploiting new tools for iron bio-fortification of rice. *Biotechnol.Adv.* **31**:1624-1633.
- BERHANU D. BEKELE. 2012. Identification and validation of molecular markers for zinc content in grains of rice (*Oryza sativa* l.) grown under aerobic condition. Ph.D. thesis, University of Agricultural Sciences, GKVK, Bangalore.
- BIRADAR, H., BHARGAVI, M. V., SASALWAD, R., PARAMA, R., AND HITTALMANI, S., 2007, Identification of QTL associated with silicon and zinc content in rice (*Oryza sativa* L.) and their role in blast disease resistance. *Indian J. Genet.*, **67 (2)**: 105 - 109.
- BISNE, R., SARAWGI, A. K. AND VERULKAR, S. B., 2009, Study of heritability, genetic advance and variability for yield contributing characters in rice. *Bangladesh J. Agril. Res.*, **34 (2)**: 175 - 179.
- BOTSTEIN, D., WHITE, R. L., SKOLNICK, M. AND DAVIS, R. W., 1980, Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.*, **32**: 314 - 331.
- BOUIS, H. E., 2003, Genetically modified food crops and their contribution to human nutrition and food quality. *Trends Food Sci. Technol.*, **14**: 191 - 209.
- BOUMAN, B. A. M. AND TUONG, T. P., 2001, Field water management to save water and increase its productivity in irrigated rice. *Agric. Water Manage.* **49**: 11-30.
- BOUMAN, B. A. M., PENG, A. R., CASTANEDA. AND VISPERAS, R. M., 2005, Yield and water use of irrigated tropical aerobic rice systems. *Agric. water manage.*, **74**: 87-105.

- BOUMAN, B. A. M., XIAOGUANG, Y., HUAQI, W., ZHIMING, W., JUNFANG, Z., CHANGGUI, W. AND BIN, C., 2002, Aerobic rice (Han Dao): A new way of growing rice in water-short areas. In: proceedings of the 12th international soil conservation organization conference, May 26-31, May, Beijing, China. Tsinghua University press. 175-181.
- BROADLEY, M. R., WHITE, P. J., HAMMOND, J. P., ZELKO, I. AND LUX, A., 2007, Zinc in plants, *New Phytol.*, **173**: 677 – 702.
- BURTON, G. M. AND DE VANE, E. M., 1953, Estimating heritability in tall Fescue (*Festuca arandaceae*) from replicated clonal material. *Agron. J.*, **45**: 478 - 481.
- CAKMAK, I., 2008, Enrichment of cereal grains with zinc: agronomic or genetic biofortification? *Plant Soil*, **302**: 1 – 17.
- CHANDEL, G., BANERJEE, S., VASCONCELOS, M. AND GRUSAK, M. A., 2010, Characterization of the root transcriptome for iron and zinc homeostasis-related genes in indica rice (*Oryza sativa* L.). *Pl. Biochem. and Biotech.*, **19**: 1-8.
- CHANEY, R. L., 1993, Zinc phytotoxicity. In: Robson AD, ed. Zinc in soil and plants. Dordrecht, the Netherlands: Kluwer Academic Publishers, 135 – 150.
- COCHRAN, W. G, AND COX, G. M., 1957, Experimental Designs. John Wiley and Sons, Inc., New York, pp. 611.
- COLANGELO, E. P., AND GUERINOT, M. L., 2004, The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *Plant cell*, **16**:3400-3412.
- COLLARD, B. C. Y., AND MACKILL, D. J., 2008, Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Phil. Trans. R. Soc. B.*, **363**: 557 – 572.
- COLLARD, B. C. Y., JAHUFER, M. Z. Z., BROUWER, J. B. AND PANG, E. C. K., 2005, An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica*, **142**: 169 – 196.
- COURTNEY, M., MCHARO, M. AND LA BONTE, D., 2008, Heritability Estimates for Micronutrient Composition of Sweetpotato Storage Roots. *Hort Sci.*, **43** (5): 1382 – 1384.
- CURIE C., Z. PANAVIENGE, C.LOULERGUE, S.L.DELLAPORTA, J.F.BRIAT AND E.L.WALKER, 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe (III) uptake. *Nature*. 409: 346 –349

- DIETARY ALLOWANCES FOR AMERICANS, Dept. of Health and Human Services and US Dept. of Agriculture, Maryland, USA, 2005, 5th edn.
- DOUCHKOV, D., HERBIK, A., KOCH, G., MOCK, H. P., MELZER, M., STEPHAN, U. W. AND BAUMLEIN, H., 2002, Nicotianamine synthase: gene isolation, gene transfer and application for the manipulation of plant iron assimilation. *Plant Soil*, **241**: 115–119.
- DOYLE, J. J., AND DOYLE, J. L., 1990, Isolation of plant DNA from fresh tissue. *Focus*, **12**: 13 - 15.
- EL-MALKY, M., EL-HABASHY, M. AND ABDELKHALIK, A. F., 2008, Rice germplasm evaluation for agronomic traits and their influence on stem borer (*Chilo Agamemnon* Bles.) resistance. *J. Agric. Res.*, **46** (3): 206 - 212.
- FALCONER, D. C., 1960, Introduction to quantitative genetics. Ronald Press, New York.
- FAROOQ, S., AND AZAM, F., 2002, Molecular markers in plant breeding - II. Some pre-requisites for use. *Pak. J. Biol. Sci.*, **5** (10): 1141 - 1147.
- FISHER, R. A., IMMER, F. R., AND TEDIN, O., 1932, The genetical interpretation of statistics of the third degree in the study of quantitative inheritance. *Genetics*, **17**: 107 - 124.
- GANGASHETTY, P.I., P.M. SALIMATH, N.G.HANAMARATT, 2013, Genetic Variability Studies in Genetically Diverse non-basmati Local Aromatic Genotypes of Rice (*Oryza sativa* L.), *Rice Genomics Genet*, **4**: 4-8.
- GARCIA-OLIVEIRA, A. L., LUBIN, T., YONGCAI, F. AND CHUANQING, S., 2009, Genetic Identification of quantitative trait loci for contents of mineral nutrients in rice grain. *J. Integr. Plant Biol.*, **51** (1): 84 - 92.
- CHANDEL G., SAMUEL, M. DUBEY, R. MEENA, 2011. In silico expression analysis of QTL specific candidate genes for grain micronutrient (Fe/Zn) content using ESTs and MPSS signature analysis in rice (*Oryza sativa* L.), *J Plant Genet Transgenics*. **2**: 11-22.
- GOTO, F., YOSHIHARA T., SHIGEMOTO N., TAKAIWA T. S., 1999, Iron fortification of rice seed by the soybean ferritin gene. *Nature Biotechnol.*, **17**: 282 - 286.
- GOVINDARAJ, M., SELVI, B., RAJARATHINAM, S. AND SUMATHI, P., 2011, Genetic variability and heritability of grain yield components and grain mineral concentration in India's pearl millet (*Pennisetum glaucum* (L) R. Br.) accessions. *Afr. J. food Agric. Nut. dev.*, **11** (3): 4758 - 4771.

- GOWDA, V. R. P., HENRY, A., YAMAUCHI, A., SHASHIDHAR, H. E. AND SERRAJ, R., 2011, Root biology and genetic improvement for drought avoidance in rice, *Field Crops Res.*, **122**: 1-13.
- GRAHAM R. D., D. SENADHIRA, S. BEEBE, C. IGLESIAS AND I. MONASTERIO, 1999, Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. *Field Crops Res.*, **60**: 57 – 80.
- GREGORIO G. B., D. SENADHIRA, R. D. GRAHAM, T. AND HTUT., 1999, improving iron and zinc value of rice for human nutrition. *Agric. Dev.*, **23**: 68 – 81.
- GROSS, J., STEIN, R. J., FETTO-NETO, A. G. AND FETT, J. P., 2003, Iron homeostasis related genes in rice. *Genet. Mol. Biol.*, **26(4)**: 477-497.
- GRUSAK, M. A. AND DELLAPENNA, D., 1999, Improving the nutrient composition of plants to enhance human nutrition and health. *Annu. Rev. Plant Physiol. Mol. Biol.*, **50**: 133 – 161.
- GUERINOT, M. L., AND YI, Y., 1994, Iron; Nutritious, noxious, and not readily available. *Plant physiol.*, **104**:815-820.
- HALLAUER, A. R., AND CARENA, M. J., 2009, Maize breeding. In: Carena M. J. (Ed.). *Handbook of Plant Breeding: Cereals*. Springer, New York. pp. 3 - 98.
- HANSEN, N C., HOPKINS, B.G., ELLSWORTH, J.W. AND JIOOEY,V.D.,2006,Iron nutrition in field crops In: *Barton LL,Albadia J (eds)*, Iron nutrition in plants and Rhizospheric Microorganisms.Pp.23-59.
- HANSON, C. H., ROBINSON, H. F., AND COMSTOCK, R. E., 1956, Biometrical studies of yield in segregating population of Korean Lespedeza. *Agron.J.*, **48**: 267-282.
- HASEGAWA, H., RAHMAN, A., SAITU, K., KOBAYASHI, M. AND OKUMURA, C., 2011, Influence of chelating ligands on bioavailability and their effect on radish growth. *Envior Exper Bot.*, **71**: 345-351.
- HIGUCHI, K., SUZUKI, K., NAKANISHI, H., YAMAGUCHI, H., NISHIZAWA, N. K. AND MORI, S., 1999, Cloning of nicotianamine synthase genes, novel genes involved in the biosynthesis of phytosiderophores. *Plant physiol.*, **119**: 471–479.
- HUKE, R. E. AND HUKE, E. H., 1997, Rice area by type of culture. South, Southeast, and East Asia. A revised and updated database. International Rice Research Institute, Los Banos, Philippines.

- INOUE, H., HIGUCHI, K., TAKAHASHI, M., NAKANISHI, H., MORI, S. AND NISHIZAWA, N. K., 2003, Three rice nicotianamine synthase genes, OSNAS1, OSNAS2 and OSNAS3 are expressed in cells involved in long-distance transport of iron and differentially regulated by iron. *Plant J.* **36**: 366–381.
- INOUE, H., KOBAYASHI, T., NOZOYE, T., TAKHASHI, M., KAKEI, Y., SUZUKI, K., NAKAZONO, M., NAKANISHI, H., MORI, S. AND NISHIZAWA, N. K., 2008, Rice OsYSL15 is an iron-regulated iron (iii)-deoxymugenic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. *The J. biol. chem.*, **284**: 3470-3479.
- ISHIMARU, Y., SUZUKI, M., TSUKAMOTO, T., SUKUKI, K., NAKAZONO, M., KOBAYASHI, T., WADA, Y., WATANABE, S., MATSUHASHI, S., TAKAHASHI, M., NAKANISHI, H., MORIS, S., AND NISHIZAWA, N. K., 2006, Rice plants take up iron as Fe^{3+} phytosiderophore and as Fe^{2+} . *The Plant. J.*, **45**: 335-346.
- JEONG, J. AND CONNOLLY, E. L., 2009, Iron uptake mechanisms in plants: functions of the fro family of Ferric reductases. *Plant Science*, **176**: 709-714.
- JOHNSON, H.W., ROBINSON, H.I. AND COMSTOCK, R.E., 1955, Estimation of genetic and environmental variability in soybean. *Agron. J.*, **47**: 314 - 318.
- JORDAN, S. A., HUMPHRIES, P., 1994, Single nucleotide polymorphism in exon 2 of the BCP gene on 7q31-q35. *Hum. Mol. Genet.*, **3 (10)**: 1915.
- KIBANDA, J. M. N. and LUZI-KIHUPI, A., 2007, Influence of genetic and genotype x environment interaction on quality of rice grain. *Afr. J. Crop Sci.*, **15 (4)**: 173 – 182.
- KIM, S. A. AND GUERINOT, M. L., 2007, Mining iron: iron uptake and transport in plants. *Febs Letters*, **581**: 2273-2280.
- KOIKE, S., INOUE, H., MIZUNO, D., TAKAHASHI, M., NAKANISHI, H., MORI, S. AND NISHIZAWA, N. K., 2004, OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. *The Plant J.*, **39**: 415-424.
- KONG, S. AND LEE, J., 2010, Antioxidants in milling fraction of black rice cultivars. *Food chem.*, **120(1)**: 278-281.
- KUMAR, L. S., 1999, DNA markers in plant improvement. *An overview. Biotechnol. Adv.*, **17**: 143 - 182.

- LAFITTE, H. R., COURTOIS, B. AND ARRAUDEAU, M., 2002, Genetic improvement of rice in aerobic systems: progress from yield to genes. *Field Crops Res.*, **75**: 171-90.
- LAKSHMANA, D., BIRADAR, B. D. AND KUMAR, R. R. L., 2009, Genetic variability studies for quantitative traits in a pool of restorers and maintainers lines of pearl millet (*Pennisetum glaucum* L.). *Karnataka J Agric. Sci.*, **22** (4): 881 - 882.
- 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.
- LU, K., LI, L., ZHENG, X., ZHANG, Z., MOU, T., AND HU, Z., 2008, Quantitative trait loci controlling Cu, Ca, Zn, Mn and Fe content in rice grains. *J. Genet.*, **87** (3): 304 - 310.
- LUCCA, P., HURRELL, R. AND POTRYKUS, I., 2001, Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. *Theor. Appl. Genet.*, **102**: 392-39.
- LUSH, J. L., 1949, Inter size correlation, regression of offspring on dams as a method of estimating heritability of characters. *Proc. Amer. Soc. Anim. Pro.*, **33**: 293 - 301.
- MACKILL, D. J., ZHANG, Z. AND REDONA, E. D., 1995, Comparison of AFLP, microsatellite and RAPD marker polymorphism in rice. *Rice Genetics Newsletter*, **12**: 245 - 247.
- MARSCHNER, H., 1995, Mineral nutrition of higher plants. Academic Press, London, PP.1 – 889.
- MARTINEZ, C. P., BORRERO, J., CARABALI, S. J., SANABRIA, Y., GIRALDO, O. X., DUQUE, M. C., SILVA, J., BARRONA, J., AND TOHME, J., 2007, Breeding strategies to increase the content of iron and zinc in the rice grain. Annu. Report 2007.
- MASUDA, H., USUDA, K., KOBAYASHI, T., ISHIMARU, Y., KAKEI, Y., TAKAHASHI, M., HIGUCHI, K., NAKANISHI, H., MORI, S. AND NISHIZAWA, N. K., 2009, Overexpression of the barley nicotianamine synthase gene hvnas1 increases iron and zinc concentrations in rice grains. *Rice*, **2**: 155-166.
- MATSUMOTO, T., WU, J. AND KANAMORI, H., et al., 2005, “The map-based sequence of the rice genome,” *Nature*, **436** (7052): 793 – 800.

- MIZUNO, D., HIGUCHI, K., SAKAMOTO, T., NAKANISHI, H., MORI, S. AND NISHIZAWA, N. K., 2003, Three nicotianamine synthase genes isolate from maize are differentially regulated by iron nutritional status. *Plant physiol.*, **132**: 1989–1997.
- MOOSE, S. P., AND MUMM, R. H., 2008, Molecular plant breeding as the foundation for 21st century crop improvement. *Plant Physiol.*, **147**: 969 – 977.
- MORGANTE, M. AND OLIVIEVI, A. M., 1992, PCR amplified microsatellites as markers in plant Genetics.
- MURTAZA, N., KITAOKA, M. AND ALI, G. M., 2005, Genetic differentiation of cotton cultivars by polyacrylamide gel electrophoresis. *J. Central Europe Agriculture*, **6**: 69 - 76.
- NAGARATHNA, T. K., SHANKAR, A. G. AND UDAYAKUMAR, M., 2010, Assessment of genetic variation in zinc acquisition and transport to seed in diversified germplasm lines of rice (*Oryza sativa* L.). *J. Agri. Technol.*, **6**: 171 - 178.
- NAGESH, RAVINDRABABU, V., USHARANI, G. AND REDDY, T. D., 2012, Grain iron and zinc association studies in rice (*Oryza sativa* L.) F1 progenies. *Arch. Appl. Sci. Res.*, **4** (1): 696 – 702.
- NISHIZAWA, N. K., 2005, The uptake and translocation of minerals in rice plants. Rice is life: Scientific perspectives for the 21st century proceedings, PP.90 – 93.
- NOZOYE, T., INOUE, H., TAKAHASHI, M., ISHIMARU, Y., NAKANISHI, H., MORI, SATOSHI. AND NISHIZAWA, N. K., 2007, The expression of iron homeostasis-related genes during the rice germination. *Plant Mol. Biol.*, **64**: 35-47.
- OGO, Y., LTAI, R. N., NAKANISHI, H., INOUE, H., KOBAYASHI, T., SUZUKI, M., TAKAHASHI, M., MORI, S. AND NISHIZAWA, N. K., 2006, Isolation and characterization of IRO2, a novel iron-regulated BHLH transcription factor in graminaceous plants. *J. Exp. Bot.*, **57**: 2867-2878.
- OGO, Y., LTAI, R. N., NAKANISHI, H., KOBAYASHI, T., TAKAHASHI, M., MORI, S. AND NISHIZAW, N. K., 2007, The rice BHLB protein OsIRO2 is an essential regulator of the genes involved in Fe uptake under Fe-deficient conditions. *The Plant J.*, **51**: 366-377.
- ORTIZ-MONASTERIO, J. L. AND GRAHAM, R. D., 2000, Breeding for trace minerals in wheat. *Food Nutr Bull.*, **21**: 392 – 396.

- PANDIAN, S. S., ROBIN, S., VINOD, K. K., RAJESWARI, S., MANONMANI, S., SUBRAMANIAN, K.S., SARASWATHI, R. AND KIRUBHAKARAN, A. P. M., 2011, Influence of intrinsic soil factors on genotype-by-environment interactions governing micronutrient content of milled rice grains. *Aust. J. Crop Sci.*, **5**: 1737 - 1744.
- PANSE, V. G. AND SUKATHME, P.V., 1985, Statistical methods for agricultural workers, ICAR, New Delhi, p145.
- PARAN, I. AND MICHELMORE, R. W., 1993, Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.*, **85**: 985 - 993.
- PARTHASARATHI, T., VANITHA, K., LAKSHAMANAKUMAR, P. AND KALAIYARASI, D., 2012. Aerobic rice-mitigating water stress for the future climate change. *Intl. J. Agron. Plant. Prod.*, **3(7)**: 241–54.
- PFEIFFER W. H. AND B. MCCLAFFERTY., 2007, Harvest plus: Breeding crops for better nutrition. International plant breeding symposium. *Crop Sci.*, **47**: 88 - 105.
- RABOY V., DICKINSON, D. B. AND BELOW, F. E., 1984, Variation in seed total phosphorus, phytic acid, zinc, calcium, magnesium, and protein among lines of Glycine max and G. soja. *Crop Sci.*, **24**: 431 – 434.
- RAJU, C. H. S., RAO, M. V. B. AND SUDARSHANAM, A., 2004, Genetic analysis and character association in F2 generation of rice. *Madras Agric. J.*, **91**: 66 - 69.
- RANA, M. K. AND BHAT, K. V., 2004, A comparison of AFLP and RAPD markers for genetic diversity and cultivar identification in cotton. *J. Plant Biochem. Biotechnol.*, **13**: 19 - 24.
- RAVINDRA BABU V., K.D. SHREYA, D. KULDEEP SINGHI, G. USHARANI AND P. NAGESH, 2012. Genetic variability studies for qualitative and quantitative traits in popular rice (*Oryza sativa* L.) hybrids of India. International journal of scientific and research publications, **2**: 1-5.
- RAVINDRA BABU V., K.D. SHREYA, D. KULDEEP SINGHI, G. USHARANI AND P. NAGESH, 2013. Evaluation of popular rice (*Oryza sativa* L.) hybrids for quantitative, qualitative and nutritional aspects. International journal of scientific and research publications, **1** - 8.
- RAVINDRA BABU V., NEERAJA, C. N. LONGVAH, T., SUREKHA, K., GANGAIAH, B., SHOBHA RANI, N. AND VIRAKTAMATH, B. C., 2011, Improvement of zinc content in rice grains through conventional and molecular breeding. Paper presentation on 3rd international zinc symposium, improving crop production and human health Hyderabad, India.

- RAVINDRA, B. V., 1996, Study of genetic parameters, correlations and path co-efficient analysis of rice under saline conditions. *Ann. Agric. Res.*, **17**: 370 - 374.
- ROBINSON H. F., R.E. COMSTOCK AND P. HARVEY. 1966. Quantitative genetics in relation to breeding on the centennial of Mendelism. *Indian J. Genet*, 26: 171 – 177.
- ROMHELD., 1987, Different strategies for iron acquisition in higher plants. *Physiol. Plantarum*, **70**: 321-334.
- ROTHSCHILD, M. F. AND SCOLLER, M., 1997, Candidate gene analysis to detect traits of economic importance in domestic livestock, *probe*, **8**: 13.
- SADEGHZADEH, B., RENGEL, Z., LI, C. AND YANG, H., 2010, Molecular marker linked to a chromosome region regulating seed Zn accumulation in barley. *Mol Breeding*, **25**: 167 – 177.
- SAMAK N. R. A., HITTALMANI, S., SHASHIDHAR, N. AND BIRADAR, H., 2011, Exploratory studies on genetic variability and genetic control for protein and micronutrient content in F₄ and F₅ generation of rice (*Oryza sativa* L.). *Asian J. Plant Sci.*, **10**: 376 - 379.
- SANDHU, N., JAIN, S., KUMAR, A., SINGH, B., AND JAIN, R., 2013, Genetic variation, linkage mapping of QTL and correlation studies for yield, root, and agronomic traits for aerobic adaptation. *BMC Genetics*, 14:104.
- SAUTTER, C., POLETTI, S., ZHANG, P. AND GRUISSEM, W., 2006, Biofortification of essential nutritional compounds and trace elements in rice and cassava. *Proc. Nut. Soc.*, **65**: 153 - 159.
- SCHMIDT, W., 2003, Iron solutions: acquisition strategies and signaling pathways in plants. *Trends Plant Sci.*, **8**(4): 188-193.
- SELVARAJ, I C., P. NAGARAJAN, K. THIYAGARAJAN, M. BHARATHI AND R. RABINDRAN, 2011, Genetic parameters of variability, correlation and path-coefficient studies for grain yield and other yield attributes among rice blast disease resistant genotypes of rice (*Oryza sativa* L.), *African Journal of Biotechnology* **10**: 3322-3334.
- SEYOUM, M., ALAMEREW, S., BANNTTE, K., 2012, Genetic variability, heritability, correlation coefficient and path analysis for yield and yield related traits in upland rice. *Plant Sci*, 1816-4951.
- SHASHIDHAR, H. E., 2007, Aerobic rice: An Efficient Water Management Strategy for Rice Production. *Food and water security in developing countries*. Chapter 12. pp. 131 – 139.

- SHIVASUBRAMANIAN, S. AND MENON, M., 1973, Heterosis and inbreeding depression in rice. *Madras Agric. J.*, 60: 1339.
- SHUKLA, V. S., SINGH, S., SINGH, S. K. AND SINGH, H., 2005, Estimation of genetic variability, heritability and genetic advance in new plant type tropical japonica rice under upland environment. *Agric. Sci. Digest*, **25**: 207 - 209.
- SINGH, B., SENTHIL NATESAN, K. A., SINGH, B. K. AND USHA, K., 2005, Improving zinc efficiency of cereals under zinc deficiency. *Curr. sci.*, **88** (1): 36 – 44.
- SINGH, S., MISHRA, S. AND KALPANA, K., 2010, Phenotypic diversity in grain yield and nutritional traits of rice (*Oryza sativa* L.). *Pest sci. & manag.*, 1- 4.
- SINHA, S.K., TRIPATI, A.K., BISEN, U.K. (2004), Study of genetic variability and correlation co-efficient analysis in mid land landraces of rice. *Annals, of Agri. Research*. 25 (1): 1-3.
- SPEROTTO, R. A., BOFF, T., DUARTE, G. L., SANTOS, L. S., GRUSAK, M. A. AND FETT, J. P., 2010, Identification of putative target genes to manipulate Fe and Zn concentrations in rice grains. *J. Pl. Physiol.*, **167**: 1500-1506.
- STEIN, A. J., NESTEL, P., MEENAKSHI, J. V., QAIM, M., SACHDEV, H. P. S. AND BHUTTA, Z. A., 2007, Plant breeding to control zinc deficiency in India: how cost-effective is biofortification? *Pub Health Nutr.*, **10**: 492 – 501.
- STEIN, R. J., RICACHENEVSKY, F. K. AND FETT, J. P., 2009, Differential regulation of the two rice ferritin genes (*OSFER1* and *OSFER2*). *Plant Science*, **177**: 563-569.
- SUMAN, A., GOURI SHANKAR, V., SUBBA RAO, L.V. AND SREEDHAR, N., 2005, Variability, heritability and genetic advance in rice (*Oryza sativa* L.). *Crop Res.*, **30**: 211 - 214.
- SUSANTO, U., 2009, Mapping QTLs controlling iron and zinc contents in polished rice grains using SSR, Poster presentation on International Rice Genetic Symposium, Manila, Philippines.
- TAKAHASHI, M., 2003, Overcoming Fe deficiency by a transgenic approach in rice. *Plant Cell, Tiss Org.*, **72**: 211–22.
- THAKUR, S.B., GHIMIRE, S.K., CHAUDHARY N.K., SHRESTHA, S.M., AND MISHRA, B., 2013, Determination of Relationship and Path Co-efficient between Pod Yield and Yield Component Traits of Groundnut Cultivars. *Nepal Journal of Science and Technology.*, **14**(2):1-8.

- TIWARI, V. K., RAWAT, N., CHHUNEJA, P., NEELAM, K., AGGARWAL, R., RANDHAWA, G. S., DHALIWAL, H. S., KELLER, B. AND SINGH, K., 2009, Mapping of quantitative trait loci for grain iron and zinc concentration in diploid A genome wheat. *J. Heredity*, **100**: 771 - 776.
- VENUPRASAD, R., SHASHIDHAR, H. E., HITTALMANI, S. AND HEMAMALINI, G. S., 2002, Tagging quantitative trait loci associated with grain yield and root morphological traits in rice (*Oryza sativa* L.) under contrasting moisture regimes. *Euphytica*, **128**: 293 – 300.
- VERT, G., GROTZ, N., DEDALDECHAMP, F. GPYMARD, F., GUERINOT, M. L., BRAIT, J. AND CURIE, C., 2002, IRT1, An Arabidopsis transporter essential for iron uptake from the soil and for plant growth. *Plant Cell*, **14**: 1223-1233.
- VERULKAR, S. B., MANDAL, N. P., DWIVEDI, J. L., SINGH, B. N., SINHA, P. K., MAHATO, R. N., DONGRE, P., SINGH, O. N., BOSE, L. K., SWAIN, P., ROBIN, S., CHANDRABABU, R., SENTHIL, S., JAIN, A., SHASHIDHAR, H. E., HITTALMANI, S., VERA CRUZ, C., PARIS, T., RAMAN, A., HAEFELE, S., SERRAJ, R., ATLIN, G. AND KUMAR, J, A., 2010, Breeding resilient and productive genotypes adapted to drought-prone rain fed ecosystem of India. *Field Crops Res.*, **117**:197–208.
- VIJAYKUMAR, C. H. M., VELETI, S. R., RAE, K. V., RAMESHA, M.S., VIRAKTAMATH, B. C. AND MISHRA, B., 2006, Breeding for high yielding rice (*Oryza sativa* L.) varieties and hybrids adapted to aerobic (non-flooded, irrigated) conditions i.e preliminary evaluation of a large number of improved germplasm lines. *Indian J. Genet.*, **66**(2): 113-118.
- VINOD, M. S., SHARMA, N., MANJUNATH, K., KANBAR, A., PRAKASH, N. N. AND SHASHIDHAR, H. E., 2006, Candidate genes for drought tolerance and improved productivity in rice (*Oryza sativa* L.). *J. Biosci.*, **31**: 101-106.
- VIRK P., G. BARBY AND H. BOUIS, 2007. Genetic enhancement for the nutritional quality of rice: proceedings of the *26th International rice research conference*, 9-12 October 2006, New Delhi, India. Printed by Macmillan India Ltd.pp.279-285.
- VIRK, P. AND BARRY, G., 2007, Biofortified rice – towards combating human micronutrient deficiencies. IRRI.
- VOS, P., HOGERS, R., BLEEKER, M., REIJANS, M., VAN DE LEE, T., HORNES, M., FRIJTERS, A., POT, J., PELEMAN, J., KUIPER, M., ZABEAU, M., 1995, AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.*, **23**: 4407 - 4414.

- WANG, X., WU, P., XIA, M., WU, Z., CHEN, Q. AND LIU, F., 2002, Identification of genes enriched in rice roots of the local nitrate treatment and their expression patterns in split-root treatment. *Gene*, **297**(1-2):93–102.
- WELCH, R. M. AND GRAHAM, R. D., 2004, Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.*, **55**: 353 - 64.
- WELCH, R. M., 2001, Impact of mineral nutrients in plants on human nutrition on a worldwide scale. In *Plant Nutrition – Food Security and Sustainability of Agroecosystems* (eds Horst, W. J. *et al.*), Kluwer, Dordrecht, The Netherlands, pp. 284 – 285.
- WHITE, P.T., 1994. Rice: the essential harvest. *Natl.Geog.*, pp: 48-79.
- WILLIAMS, J. G. K., KUBLELIK, A. R., LIVAK, K. J., RAFALSKI, J. A., TINGEY, S. V., 1990, DNA polymorphism's amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.*, **18**: 6531 - 6535.
- WORLD HEALTH ORGANIZATION, 1996, Trace elements in human nutrition and health, WHO, Geneva, Switzerland.
- WORLD HEALTH ORGANIZATION, 2002, Zinc for better health: The world health report: Geneva, Switzerland.
- WORLD HEALTH ORGANIZATION, 2011, Use of multiple micronutrient powders for home fortification of foods consumed by infants and children 6–23 months of age. Geneva.
- www.aerobicrice.in
- www.behealthyandwell.com
- www.chinese-black-rice.com
- www.iza.com/zinc_health.html
- www.rice2004.org
- ZENG, Y., SHEN, S., WANG, L., LIU, J., PU, X., DU, J. AND QIU, M., 2005, Correlation of Plant Morphological and Grain Quality Traits with Mineral Element Contents in Yunnan Rice. *Rice Sci.*, **12** (2): 101 - 106.
- ZHANG, X., ZHANG, G., GUO, L., WANG, H., ZENG, D., DONG, G., QIAN, Q. AND XUE, D., 2011, Identification of quantitative trait loci for Cd and Zn concentrations of brown rice grown in Cd-polluted soils. *Euphytica*, **180**: 173 - 179.

- ZIETKIEWICZ, E., RAFALSKI, A. AND LABUDA, D., 1994, Genome fingerprinting by simple sequence repeats (SSR)-anchored PCR amplification. *Genomics*, **20**: 176 - 183.
- ZIMMERMANN, M. B. AND HURRELL, R. F., 2002, Improving iron, zinc and vitamin a nutrition through plant biotechnology. *Curr. Opinion Biotech.*, **13**: 142-145.
- ZOZALI, M. A., SHOKRZADESH, M., BAZERAFSHAN, E., HAZRATI, M., AND TAVAKKOULI, A., 2006, Investigation of zinc content in Iranian rice (*Oryza sativa* L.) and its weekly intake. *Am-Euras. J. Agric. Envir. Sci.*, **1** (2): 156 - 159.