

## A COMPARATIVE ANALYSIS OF UPTAKE AND ACCUMULATION PATTERN OF IRON IN INDIAN BARNYARD MILLET AND RICE

V VIJAYPRABHA<sup>1#</sup>, S VARANAVASIAPPAN<sup>\*#</sup>, D SUDHAKAR, L ARUL, E KOKILADEVI,  
C VANNIARAJAN<sup>2</sup>, A SENTHIL<sup>1</sup> AND P JEYAKUMAR<sup>1</sup>

*Department of Plant Biotechnology, Tamil Nadu Agricultural University,  
Coimbatore-641003, Tamil Nadu, India*

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### Abstract

The present study focuses on how Fe accumulation and re-mobilization pattern differs between Indian barnyard millet and rice at different growth stages. Screening Indian barnyard millet accessions resulted in identification of MDU 1, as the highest accumulator of Fe in seeds. Further, pot culture studies were conducted with MDU 1, ACM-16-5 (low seed Fe accumulating barnyard millet accession) and a rice variety (ASD 16). The accumulation pattern of Fe in the seeds of MDU 1, ACM-16-5 and ASD 16 was influenced by re-mobilization of nutrients from non-flag leaf, flag leaf, root and stem during flowering and maturity. The expression of metal homeostasis genes such as *YSL16*, *ZIP2* and *ZIP8* in leaf and root tissues of MDU 1 at the flowering stage appear to play a vital role in Fe absorption and translocation. Remobilization of nutrients from various plant parts to seeds at flowering and maturity stage is efficient in barnyard millet.

### Introduction

Plant provides necessary micro and macro nutrients to the human population. The uneven distribution of these nutrients within the plants is a major concern from human nutrition point of view. Leaves tend to accumulate more micronutrients while the edible parts such as the grains and tubers have insufficient level of nutrients. Rice leaves contain adequate amount of Fe for human consumption where as, the edible endosperm contains only a minimal quantity (Beyer 2010, Waters and Sankaran 2011). To make rice grains Fe rich, it is necessary to genetically improve the rice varieties to accumulate more Fe in endosperm. Identification of novel sources which can provide genes involved in Fe homeostasis with a view to improve Fe content in cereal grains, particularly in endosperm, would be a relevant approach to combat its widespread malnutrition (Waters and Sankaran 2011, Masuda *et al.* 2009, Kobayashi *et al.* 2019). Knowledge on the natural genetic variation for traits such as Fe uptake, transport, and sequestration within the species level is crucial for developing resources in biofortification programs (Ludwig and Slamet-Loedin 2019).

Indian barnyard millet (*Echinochloa frumentaceae* (Roxb.) Link), is one of the important minor millets known for its fast maturity, high storability, and the ability to grow on poor soils (Sood *et al.* 2015, Renganathan *et al.* 2020). In addition, barnyard millet accumulates high Fe content in seeds compared to the most widely consumed cereals such as rice, wheat and other small millets (Vinoth and Ravindhran 2017). For higher accumulation to occur, a plant should possess an effective strategy for enhanced nutrient uptake, efficient transport and sequestration mechanism (Mari *et al.* 2020). Barnyard millet is considered as a potential food crop of the future for its nutritional values such as high Fe and fiber content (Muthamilarasan and Prasad 2020, Renganathan *et al.* 2020). There are almost no genetic or genomic resources available and no comprehensive studies on Fe

\*Author for correspondence: <varanavasiappan.s@tnau.ac.in>. #Equal contribution. <sup>1</sup>Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore-641003, Tamil Nadu, India. <sup>2</sup>Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Madurai-625 104, Tamil Nadu, India.

uptake and mobilization have been conducted to date. Over the last few decades, the genes involved in Fe homeostasis in rice and Arabidopsis have been well characterized and such genes are utilized to improve Fe uptake and transport in the plants. However, in barnyard millet, specific role of metal transporters in Fe uptake and accumulation are not well characterized. The present study is an attempt to understand uptake, translocation, accumulation and re-mobilization of Fe in barnyard millet and rice at molecular level.

### Materials and Methods

Eighteen Indian barnyard millet accessions obtained from Agricultural College and Research Institute, Madurai - 625 104, Tamil Nadu, India were screened for seed Fe content. Based on the results, two contrasting genotypes MDU 1, a high Fe accumulator and ACM-16-5, a low Fe accumulator and a rice variety ASD 16 were selected for the comparative study of the Fe uptake and accumulation pattern at different growth stages. The plants were grown under controlled greenhouse conditions. The Fe content in flag leaf, non-flag leaf, stem, root and grain of MDU 1, ACM-16-5 and ASD 16 were estimated at different growth stages. The growth stages of barnyard millet were classified as seedling (15 DAS), vegetative (40 DAS), flowering (65 DAS) and maturity (90 DAS). In rice, it was classified as seedling (25 DAS), vegetative (55 DAS), flowering (85 DAS) and maturity (100 DAS). The experiments were performed with four replications. Plant samples were harvested at respective growth stages and Fe content was determined by Atomic Absorption Spectrometer (PinAAcle 900T, Perkin Elmer Inc., USA) as described by Shanmugam *et al.* (2011). All the samples were analyzed in quadruples.

Root and leaf tissues of barnyard millet and rice collected at the flowering stage were used for RNA isolation using Trizol Reagent (T9424, TRI Reagent<sup>®</sup>, Sigma-Aldrich, USA). The isolated RNA was quantified using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and then used for cDNA synthesis using the SuperScript<sup>™</sup> III First-Strand Synthesis kit (Thermo Scientific, USA, catalogue no. 18080051) and stored at -20°C for further use. Degenerate primers were designed based on conserved domains in the sequences of rice (*Oryza sp.*) and foxtail millet (*Setaria italica*). Semi-quantitative Reverse Transcription - PCR analysis of metal homeostasis genes were carried out with *Actin* as an internal control.

One way ANOVA with TUKEYS HSD was used to analyze the significance of differences in tissue Fe content across different time points between the tested lines (barnyard millet and rice). When ANOVA values were significant, means were compared by the least significant difference at the 0.01 and 0.05 probability levels.

### Results and Discussion

Plants employ a wide range of mechanisms such as altering the rate of nutrient uptake, altered root morphology and physiology, storage and re-mobilization of mineral nutrients between organelles, cells, tissues, and organs to enrich the nutrient content in sink tissues including grains. In the present study, 18 Indian barnyard millet accessions were analyzed for Fe content in whole grain. Fe concentration ranged from 0.78 to 14.57 mg/100 g. The Fe concentration in seeds was high in MDU 1 with the values of 14.57±1.12 mg/100 g followed by CO1 (12.49±0.60 mg/100 g) and ACM-16-8 (12.31±0.98 mg/100 g) (Table 1). Barnyard millet accessions such as ACM-16-11, ACM-16-15, ACM-16-5 and ACM-12-110 were found to be low seed Fe accumulators. This might be due to the variation in their genetic architecture underlying the metal ion homeostasis. Thomas *et al.* (2016) reported that genetic variability was a factor that determines effective mineral accumulation in the edible plant parts.

**Table 1. Seed Fe content of Indian barnyard millet accessions.**

Sl. No.	Barnyard millet accession	Fe concentration (mg/ 100 g dry weight)
1	ACM-16-1	7.76 ± 0.44 <sup>bcd</sup>
2	ACM-16-3	9.16 ± 0.98 <sup>abcd</sup>
3	ACM-16-4	6.14 ± 0.03 <sup>cdef</sup>
4	ACM-16-5	1.52 ± 1.98 <sup>hij</sup>
5	ACM-16-6	6.26 ± 0.70 <sup>cdef</sup>
6	ACM-16-7	6.36 ± 0.39 <sup>cdef</sup>
7	ACM-16-8	12.31 ± 0.98 <sup>abc</sup>
8	ACM-16-9	10.60 ± 1.09 <sup>cdef</sup>
9	ACM-16-10	3.48 ± 0.23 <sup>fghi</sup>
10	ACM-16-11	0.78 ± 0.08 <sup>j</sup>
11	ACM-16-12	5.40 ± 0.17 <sup>defg</sup>
12	ACM-16-13	5.65 ± 0.35 <sup>defg</sup>
13	ACM-16-14	2.96 ± 0.10 <sup>def</sup>
14	ACM-16-15	1.01 ± 0.05 <sup>hij</sup>
15	ACM-12-110	1.77 ± 0.95 <sup>hij</sup>
16	ACM-10-082	5.73 ± 0.29 <sup>efgh</sup>
17	MDU 1	14.57 ± 1.12 <sup>a</sup>
18	CO 1	12.49 ± 0.60 <sup>ab</sup>

100 mg of each barnyard millet lines seeds were acid digested for metal estimation. Data represent mean ± SE of mean of four independent replications. Significant differences between the accessions were analyzed by one way ANOVA performed with a completely randomized design. Similar letters in column are not significantly different.

Significant variation in accumulation of Fe was noted in the roots/shoots of barnyard millet and rice at different growth stages (Fig. 1). In roots, maximum of Fe accumulation was found at the flowering stage. When compared with ASD 16 and ACM-16-5, the roots of MDU 1 absorbed more Fe except at maturity. From seedling to flowering, the Fe accumulation in roots was significantly higher in barnyard millet (both MDU 1 and ACM-16-5) than rice (Fig. 1a). Madurai 1 (MDU 1) accumulated higher root Fe content in all the tested stages except maturity (Fig. 1a). A well coordinated Fe up take machinery appears to play a role in MDU 1 to improve the Fe content in root tissues. At flowering stage, *ZIP2*, member of ZRT-IRT1-like proteins, exhibited higher expression in the root tissues of MDU 1 compared to ASD 16 (Fig. 3b). *ZIP2* mediates the uptake of zinc (Zn) and manganese (Mn) in plants (Milner *et al.* 2013). The expression of *IRT1* and *IRT2* are similar both in barnyard millet and rice. Iron-regulated transporter 1 (*IRT1*) and *IRT2* are well characterized transporters and regulated by Fe status (Vert *et al.* 2002, Vert *et al.* 2009, Shanmugam *et al.* 2013). The expression of nicotinamine synthase1 and 2 (*NAS1* and *NAS2*) were observed in the roots of barnyard millet and rice. The expression of *NAS2* facilitates the long-distance transport of Fe in plants (Inoue *et al.* 2003). Nicotinamine (NA), a chelator of metal cations such as Fe<sup>2+</sup> and Zn<sup>2+</sup> helps in enhanced uptake and transport of Fe and Zn (Masuda *et al.* 2009, Nishiyama *et al.* 2012). In

MDU 1, further detailed studies are needed to understand the complex network associated with Fe uptake and metal homeostasis.

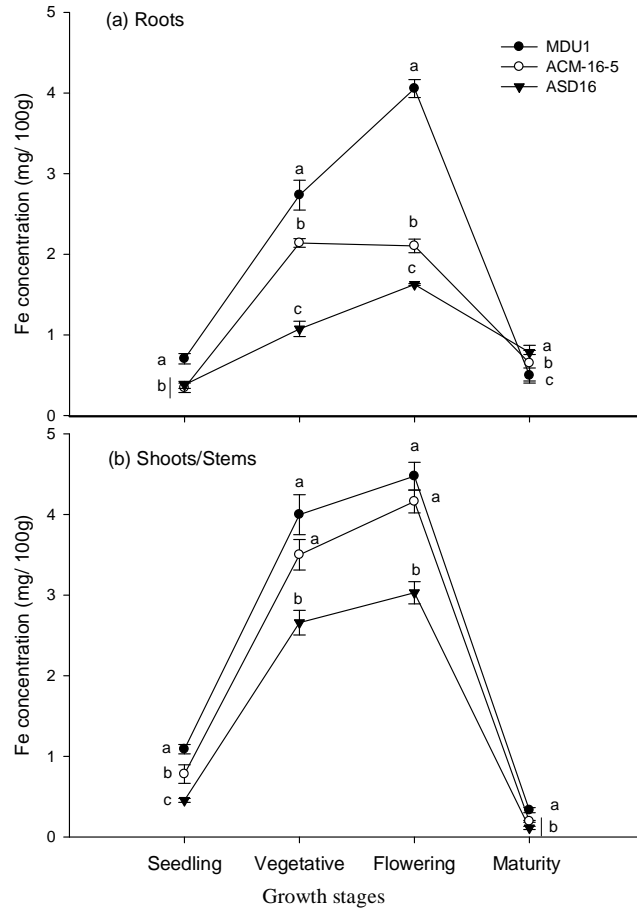


Fig. 1. Fe accumulation pattern in roots and shoots of barnyard millet and rice. Fe content in roots (a) and shoots/stems (b) of barnyard millet lines (MDU 1, ACM-16-5) and rice (ASD 16) at seedling, vegetative, flowering and maturity stage. Data represent mean  $\pm$  SE of mean of four independent replications. Different letters at a particular growth stage represent statistically different means at  $P < 0.05$  or  $P < 0.01$  (one-way ANOVA with a TUKEY HSD test).

Studying the changes in the ionic composition of leaves is a prerequisite to know about the net re-mobilization of nutrients from older leaves to younger leaves or to developing edible parts (Sperotto *et al.* 2012, Sperotto *et al.* 2014). Leaf Fe content analysis in barnyard millet and rice revealed that there was a gradual increase in the accumulation of Fe up to flowering phase and then a gradual decrease was found towards maturity (Figs 1 and 2). Though the nutrient accumulation trend was similar in barnyard millet and rice across different growth stages, there was a significant difference in the amount of nutrient absorbed between barnyard millet and rice. Overall, both barnyard millet lines (MDU 1 and ACM-16-5) significantly accumulated more Fe in the stem, leaf and flag leaves than rice. At maturity, in MDU 1, the net reduction in Fe content in all the tissues directly resulted in increased Fe content in grain tissues (Figs 1b and 2). The MDU 1 significantly accumulated more seed Fe both at flowering and maturity stages. Interestingly, grain Fe concentration of MDU 1 increased up to 55% from flowering to maturity, but in ASD 16, the grain

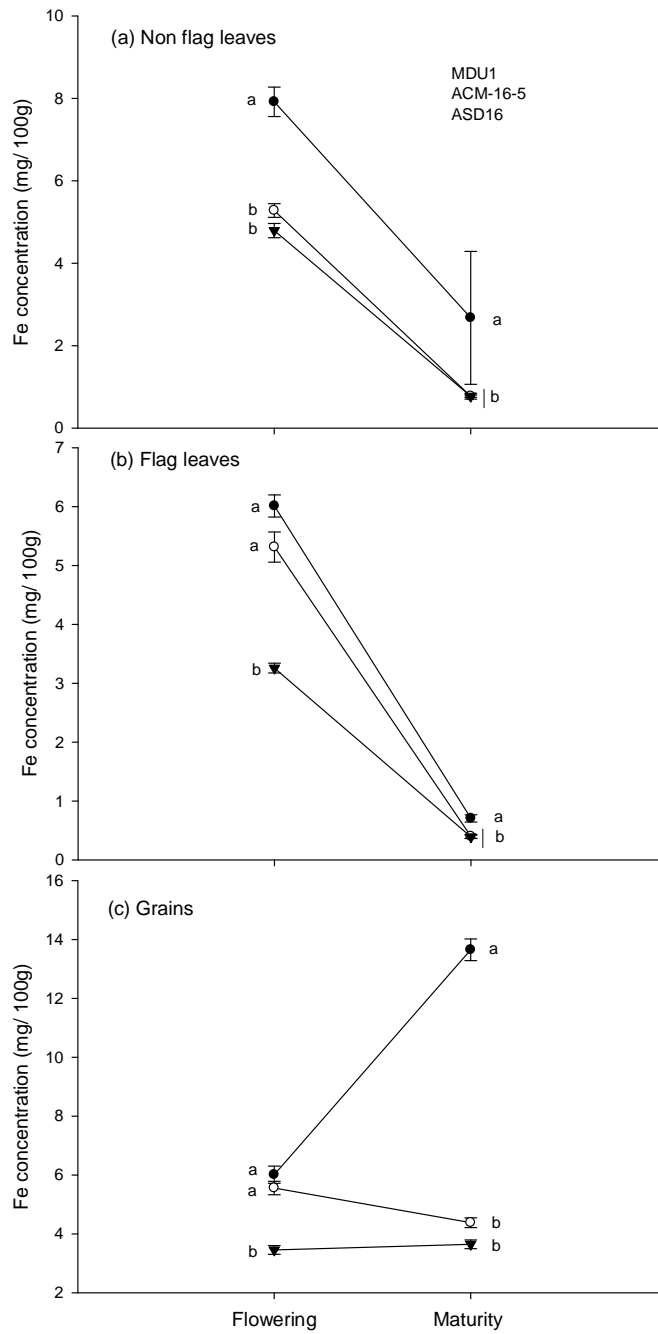


Fig. 2. Fe content in non-flag leaves (a), flag leaves (b) and grains (c) of barnyard millet lines (MDU 1, ACM-16-5) and rice (ASD 16) at flowering and maturity stages. Data represent mean± SE of mean of four independent replications. Different letters at a particular growth stage represent statistically different means at P< 0.05 or P< 0.01 (one-way ANOVA with a TUKEY HSD test).

Fe concentration increased only by 6% from flowering to maturity (Fig. 2c). Iron (Fe) partitioning is better in MDU 1 when compared to rice and ACM-16-5, the other barnyard millet accession. This stage is crucial and it determines the amount of Fe loaded into the grains from various plant parts such as leaf, flag leaf and stem tissues.

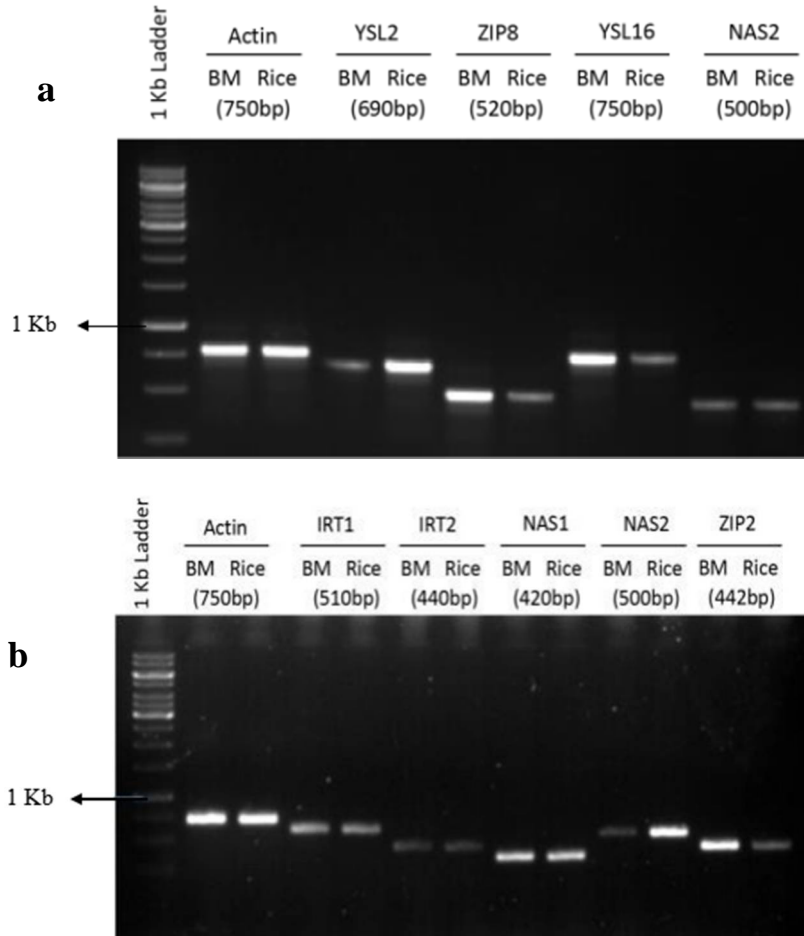


Fig. 3. Reverse-transcription polymerase chain reaction (RT-PCR) of metal homeostasis genes at flowering stage in shoots (a) and roots (b) of barnyard millet, MDU 1 (BM) and ASD 16 (Rice). The expected size of the amplicons for the analyzed genes was given in brackets. The expression of *Actin* was used as an internal control.

In MDU 1, *ZIP8* and *YSL16* expression were higher in shoots when compared to the rice variety ASD 16. In plants, *ZIP8* function as Fe and Zn transporter and up regulated by cellular Fe status (Lee *et al.* 2010, Wang *et al.* 2012). A detailed study in rice revealed that *ZIP8* is responsible for Zn uptake from the soil, translocation within the root and from root to shoot and as well as for the storage in grains under Zn deficiency (Lee *et al.* 2010). The higher expression of *YSL16* in leaf tissue of MDU 1 appears to be involved in enhanced Fe accumulation. This could be substantiated with the findings of Lee and coworkers (Lee *et al.* 2012) that *OsYSL16*, a plasma membrane-localized transporter gene which is responsible for Fe homeostasis by distributing Fe within the plant system irrespective of Fe-deficient or sufficient condition. Zheng *et al.* (2012) reported that *OsYSL16* was mainly expressed in vascular tissues of roots, nodes and leaves of rice. A

comparative shoot transcriptome analysis of Indian barnyard millet (*Echinochloa frumentacea*) and barnyard grass (*E. crus-galli*) showed that genes involved in Fe and Zn homeostasis including members of NAC domain containing proteins (NAC) and vacuolar protein sorting-associated proteins (VAP) are over expressed in Indian barnyard millet (Jayakodi *et al.* 2019). A thorough Fe responsive transcriptomic analysis at different stages of grain development in MDU 1 would efficiently provide deep insights into Fe accumulation and serve as a model system to unveil the molecular basis for micronutrient accumulation. The current study indicates that MDU 1 transported more Fe from source to sink tissues which resulted in high Fe density in grains. Thus, the efficient Fe mobilization from various tissues is vital for high seed Fe accumulation.

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