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Research Article

Phenotypic evaluation and gene expression analysis of metal homeostasis genes in barnyard millet (*Echinochloa frumentaceae*) lines

V. Vijay Prabha[1], S. Varanavasiappan[2], P. Jeyakumar[1], D. Sudhakar[2], M. Raveendran[2], C. Vanniarajan[3], T. Chitdeshwari[4] and A. Senthil[1]
[1]Department of Crop Physiology, Directorate of Crop Management, Tamil Nadu Agricultural University, Coimbatore - 641003, Tamil Nadu
[2]Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore - 641003, Tamil Nadu
[3]Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Madurai – 625 104, Tamil Nadu
[4]Department of Soil Science and Agricultural Chemistry, Directorate of Natural Resource Management, Tamil Nadu Agricultural University, Coimbatore - 641003, Tamil Nadu
E-Mail:varanavasiappan.s@tnau.ac.in

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Abstract

Barnyard millet, a versatile and multi-purpose crop, is nutritionally superior to other major cereals and it is known for the efficient accumulation of metals, mainly Fe. To study the phenotypic variation in different barnyard millet lines for selecting desirable phenotype with varying metal accumulation, a pot culture experiment was conducted with nineteen barnyard millet lines and screened at seedling, vegetative, flowering and maturity stages. The experiments were laid out in a completely randomized design with three replications. Out of nineteen lines screened, the increased plant height, total dry matter production, total chlorophyll content and nitrate reductase activity, was observed in the variety MDU1 and the performance of ACM-16-5 was comparatively lower. The results showed that performance of MDU1 and ACM-16-5 was significantly varied. Since, barnyard millet is an active accumulator Fe, gene expression study was carried out to unveil the transporters that are involved in Fe and Zn uptake and translocation at seedling and vegetative growth stages of MDU1, with rice (ASD16) as control. Of all six genes studied, the expression of *NAS1*, *NAS2* and *YSL2* in leaf of MDU1 at seedling and vegetative stages was variably expressed when compared to ASD16 and this could pave the way for enhanced metal uptake and its utilization in plant growth and development.

Key words
Barnyard millet, physiology, gene expression, transporters

Introduction

Barnyard millet *(Echinochloa frumentaceae)* is also known as Indian barnyard millet or billion-dollar grass. It can even be cultivated in the places of low rainfall areas where the average annual rainfall is below 350mm. It is widely grown as a cereal in India, Pakistan and Nepal (USDA-NRCS, 2002). In India, it is widely grown in most of the southern and central states of India. Barnyard millet is a multi-purpose crop mainly cultivated for food and fodder for humans and animals. Millets are unique among other cereals. They have received better attention from the health point of view due to their high dietary fibre content, low glycemic index, polyphenols, proteins, and richness in calcium (Devi *et al*., 2011). Ugare *et al*. (2011) reported that the consumption of dehulled varieties of barnyard millet lowers the risk of type 2 diabetes and cardiovascular disease. Along with its better nutritional properties of barnyard millet, it acts as an efficient accumulator of metals such as Fe and Zn.

In plants, Fe and Zn play a crucial role in maintaining proper metabolic and physiological processes. Fe acts as a co-factor in chlorophyll biosynthesis, nitrogen fixation, DNA replication, reactive oxygen species (ROS) scavenging and electron transport chain in both mitochondria and chloroplasts (Nouet *et al.*, 2001; Yruela, 2013). Chlorosis of young leaves is the most apparent visible symptom of Fe deficiency (Marschner, 1995) and its lack also triggers oxidative stress (Tewari *et al*., 2005; Bashir *et al*., 2007).

Like Fe, Zn, a divalent cation, also plays diverse roles in different cellular processes (Ishimaru *et al.*, 2011). The metabolism of proteins, nucleic acids, carbohydrates, and lipids is dependent on Zn to a larger extent. Ishimaru *et al.* (2011) reported that the tight regulation of the Zn concentration is essential for its uptake by the plants. Fukao *et al.* (2011) stated that many Fe and Zn transporters had been identified in plants so far.

Transporters are essential for the entry and exit of nutrients to the vacuole for storage or to the other organelles for functional purposes. Translocation through the xylem requires nutrient efflux from the symplast into the apoplast through transporters and subsequent influx through transporters back into the symplast of distal tissues. Also, nutrient transporters are involved in re-mobilization and transportation from senescent leaves to developing tissues or in response to nutrient limitation. These transporters might be involved in the efficient uptake and translocation of Fe and Zn in barnyard millet. A better understanding of the morpho-physiological characters can be used to identify the most suitable line among various barnyard millet lines for its improved nutritional value. Hence an experiment was designed to study the variation in morpho-physiological characters in various barnyard millet lines and also to know about differential expression of metal homeostasis genes in better performing line at early growth stages, *i.e*., seedling and vegetative stages with rice (ASD16) as control plant.

Materials and Methods

Owing to differences in the accumulation of Fe and Zn in seeds (unpublished data) of nineteen barnyard lines, a pot culture experiment was conducted to evaluate the morpho-physiological characteristics of these barnyard millet lines concerning Fe and Zn. The list of barnyard millet lines taken for the study were ACM-16-1, ACM-16-3, ACM-16-4, ACM-16-5, ACM-16-6, ACM-16-7, ACM-16-8, ACM-16-9, ACM-16-10, ACM-16-11, ACM-16-12, ACM-16-13, ACM-16-14, ACM-16-15, ACM-10-082, ACM-12-110, MDU1, CO1, and CO2. The seeds were obtained from Agricultural College and Research Institute, Madurai, Tamil Nadu. The experiment was conducted at the glasshouse, Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore with a completely randomized design and it was replicated thrice. Morpho-physiological characters such as plant height, total dry matter production, total chlorophyll content and nitrate reductase activity were recorded at seedling (15DAS), vegetative (40DAS), flowering (65DAS) and at maturity (90DAS). Besides the morpho-physiological evaluation, studying the gene expression underlying metal homeostasis and its better accumulation in better performing line in comparison with rice (ASD16) as control was done.

***Plant height*:** The height of plants was measured from the base of the stem to the tip of the plant. The average height of these plants was calculated and expressed as mean plant height (cm).
***Total dry matter production*:** The entire plant was uprooted with the plant system intact at different stages to estimate total dry matter production. Plant samples were shade dried and then oven-dried (65°C) for three days. The dry weight of the whole plant was recorded and expressed in g plant^{-1}.
***Total chlorophyll content*:** Total chlorophyll content in leaves was estimated using the method described by Hiscox and Israelstam (1949) and expressed in mg g^{-1} fresh weight.

***Nitrate reductase activity*:** Nitrate reductase activity was estimated as per the method described by Nicholas *et al.* (1976) and expressed as µg NO_2 g^{-1}hr^{-1} fresh weight.

Root and leaf tissues of barnyard millet and rice collected at seedling (15 DAS and 25 DAS for barnyard millet and rice, respectively) and vegetative stage (25 DAS and 55 DAS for barnyard millet and rice, respectively) were used for RNA isolation using Trizol Reagent (T9424, TRI Reagent®, Sigma-Aldrich, USA). The isolated RNA was quantified using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA) and then used for cDNA synthesis using the SuperScript™ III First-Strand Synthesis kit (Thermo Scientific, USA, catalogue no. 18080051) and stored at -20˚C for further use. Semi-quantitative Reverse Transcriptase PCR analysis of six selected metal homeostasis genes such as *IRT1, NAS1, NAS2, ZIP2, ZIP7* and *YSL2*were carried out with gene *Actin* as an internal control. Degenerated primers (Table 1) were designed by comparing the sequences of rice (*Oryza* sp.) and foxtail millet (*Setaria italica*) using BLAST and CLUSTAL W, multiple sequence alignment tool to find the conserved domains. PCR conditions for metal homeostasis genes were optimized with the genomic DNA of barnyard millet.

One way ANOVA was performed with a completely randomized design and their significance was tested at 1% and 5% interval ($p<0.01$, $p<0.05$).

Results and Discussion

Plant height of nineteen barnyard millet lines were recorded at different growth stages and depicted in Fig. 1. The barnyard millet line, MDU1, a high Fe and Zn accumulator (Unpublished data), achieved the highest plant height. The uptake and utilization of macro and micro-nutrient had significantly increased the plant height by accelerating the enzymatic activity and auxin metabolism in plants (Sudha and Stalin, 2015). The lowest plant height was achieved by ACM-16-5, which is a low Fe and Zn accumulator (Unpublished data). Another possible reason behind the attainment of highest plant height by MDU1was due to the presence of adequate Fe and Zn content which facilitates increased enzymatic activity and auxin metabolism that promotes better cell division and cell enlargement subsequently increasing the plant height (Bameri *et al.,* 2013; Rawashdeh and Sala 2014; Sudha and Stalin, 2015; Singh and Singh, 2018).

The quantum of dry matter produced indicates the overall utilization of available resources by the plant (Mahendar *et al.* 2017). Total dry matter production was found significantly varied among the barnyard millet lines. The superiority of MDU1 over ACM-16-5 (Fig 2) in total dry matter production at all the growth stages might be due to the enhanced photosynthetic activity and finally resulted in enhanced total dry matter production (Sunil and Sankaralingappa, 2014). Duraisamy and Mani (2001) reported that the increased dry matter production was due to the increased supply of photosynthates to sink due to higher chlorophyll content and photosynthesis, in turn, increased the total dry matter production. The reports of Barua and Saikia (2018) indicated that Zn favors the proliferation of roots, which increases the nutrient uptake from the soil and enhances the vegetative growth of the plant. Similar findings were reported by Singh and Singh (2018).

The total chlorophyll content of various barnyard millet lines at different crop growth stages was depicted in Fig. 3. A significant variation was found between the barnyard millet lines irrespective of stages. The maximum value of total chlorophyll content was recorded in MDU1, followed by CO2 and ACM-12-110. The minimum value of total chlorophyll content was observed in ACM-16-5 and ACM-16-7. Since MDU1 was the highest accumulator of Fe and Zn, its role in increased chlorophyll content was indispensable. Siavoshi and Laware (2013) stated that Fe form complexes with enzymes associated with chloroplast synthesis. Higher the Fe content in the plant system, higher will be chlorophyll synthesis and RNA metabolism in the chloroplasts. This was supported by the findings of Roosta *et al*. (2018). The less Fe content in ACM-16-5 and ACM-16-7 might have resulted in reduced chlorophyll synthesis.

Nitrate reductase is an essential enzyme in the assimilation of nitrate-nitrogen by plants (Epstein and Bloom 2004). The nitrate reductase activity was higher in MDU1 followed by ACM-16-15, CO2 and ACM-12-110. The activity was lower in ACM-16-5 at all the growth stages (Fig 4). The activity of this enzyme in MDU1, was 34%, 21%, 22%, 33% higher than ACM-16-5 at seedling, vegetative, flowering and maturity stages, respectively. Moro *et al.* (2017) reported that the decrease in Fe content affects the conversion of nitrate to nitrite and ultimately affects the nitrogen utilization by plants. Also, Fisher *et al.* (2005) and Fageria *et al.* (2014) stated that the reduction capacity of nitrate reductase enzyme in plants was majorly influenced by Fe content.

Plants involve a complex network of homeostasis mechanisms to regulate the uptake, accumulation, trafficking, and detoxification of metals (Clemens, 2001). This applies to metal hyper-accumulation also. Based on the morpho-physiological behavior of the nineteen barnyard millet lines, MDU1 performed better over other barnyard millet lines. Moreover, MDU1 was identified as the hyper-accumulator of Fe and Zn (Unpublished data). The process involved behind the hyper-accumulation in MDU1 provoked us to study the expression analysis of selected Fe and Zn homeostasis genes in leaf and root at seedling and vegetative stage in comparison with rice. The differential expression of metal homeostasis genes in the leaf tissue of barnyard millet and rice was given in Fig. 5A. The results revealed that the expression of *YSL2* was found in leaf tissue of MDU1 but not in rice. Whereas, in root tissue, the expression of NAS1 was found to be varied (Fig. 5B)

The NA-Fe (II) transporter YSL2 (Yellow Stripe Like-2), localized in leaf phloem cells and vascular bundles of flowers and developing seeds. Ishimaru *et al.* (2010) reported that the plants with reduced YSL2 function had reduced Fe concentration in the grains and this clearly indicated the role of *YSL2* in iron acquisition. Also, it was reported that YSL2 is an essential Fe-nicotinamine transporter involved in Fe translocation, particularly in shoots and endosperm. Inoue *et al.* (2003) reported that NAS1, which encodes NAS enzyme, was found in the companion cells of roots and helped in phloem loading and unloading. Additionally, the expression of other metal homeostasis genes such as *NAS2* in leaf and *ZIP7* in root tissues might contribute for enhanced Fe and Zn accumulation in MDU1.

The expression pattern of metal homeostasis genes in leaf tissue of barnyard millet and rice at the vegetative stage was given in Fig 5C. The expression of *NAS1* and *YSL2* was varied in MDU1 when compared to ASD16. Whereas in root tissue (Fig 5D) of MDU1 and ASD16, the expression of *IRT1, ZIP2* and *NAS1* were equal and those genes were reported to be involved in the enhanced Fe and Zn uptake from the soil and translocation within the plant system during the vegetative stage.

Barnyard millet lines that differ in metal accumulation were evaluated under pot culture experiment. Out of nineteen barnyard millet lines screened, MDU1, varied significantly among all the other lines in plant height, total dry matter production, total chlorophyll content, and nitrate reductase activity. The efficient use of absorbed macro and micro-nutrient from the soil by MDU1, mainly, Fe and Zn, which play a vital role in maintaining metabolic and physiological processes, could be the possible reason for its better performance over other lines. Due to the superiority of MDU1 over all the other genotype, the genetic architecture underlying the metal uptake and translocation was further analyzed. The expression of key metal homeostasis genes such as *NAS1*, *YSL2*, *NAS1*, *ZIP2*, *ZIP7* and *IRT1* helps in effective Fe and Zn acquisition from the soil and its utilization for proper plant growth and development.

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Table 1. List of degenerate primers used for semi-quantitative real time PCR

Name of the gene	Primer sequences		Amplicon size (bp)
	5’ primer	3’primer	
Actin	TGTGATGTTGATATCAGGAAGGA	GGGACCGGTTTCGTCATACT	750
IRT1	TCGTCAAGGCSTTCGCGTC	CCGATCACCACCGAGTGCAC	510
NAS1	AGCAAGCTGGAGTACGASCT	GATCGACGATCGGGTASAGGAA	420
NAS2	CTSTTCACCGASCTCGTCA	TCCAYCTTGCAGCACTTGCA	500
YSL2	TATCCAAGTGGGACTGCAACTG	CCCATGATCARAGCTACACACA	690
ZIP2	TGCTTYCACTCCGTCTTCGA	AGGTGTCCCATATCATGACGAC	442
ZIP7	GTGTKCACTCKGTGATCAT	GCCCAKATTGCAAGNGATGACAT	520

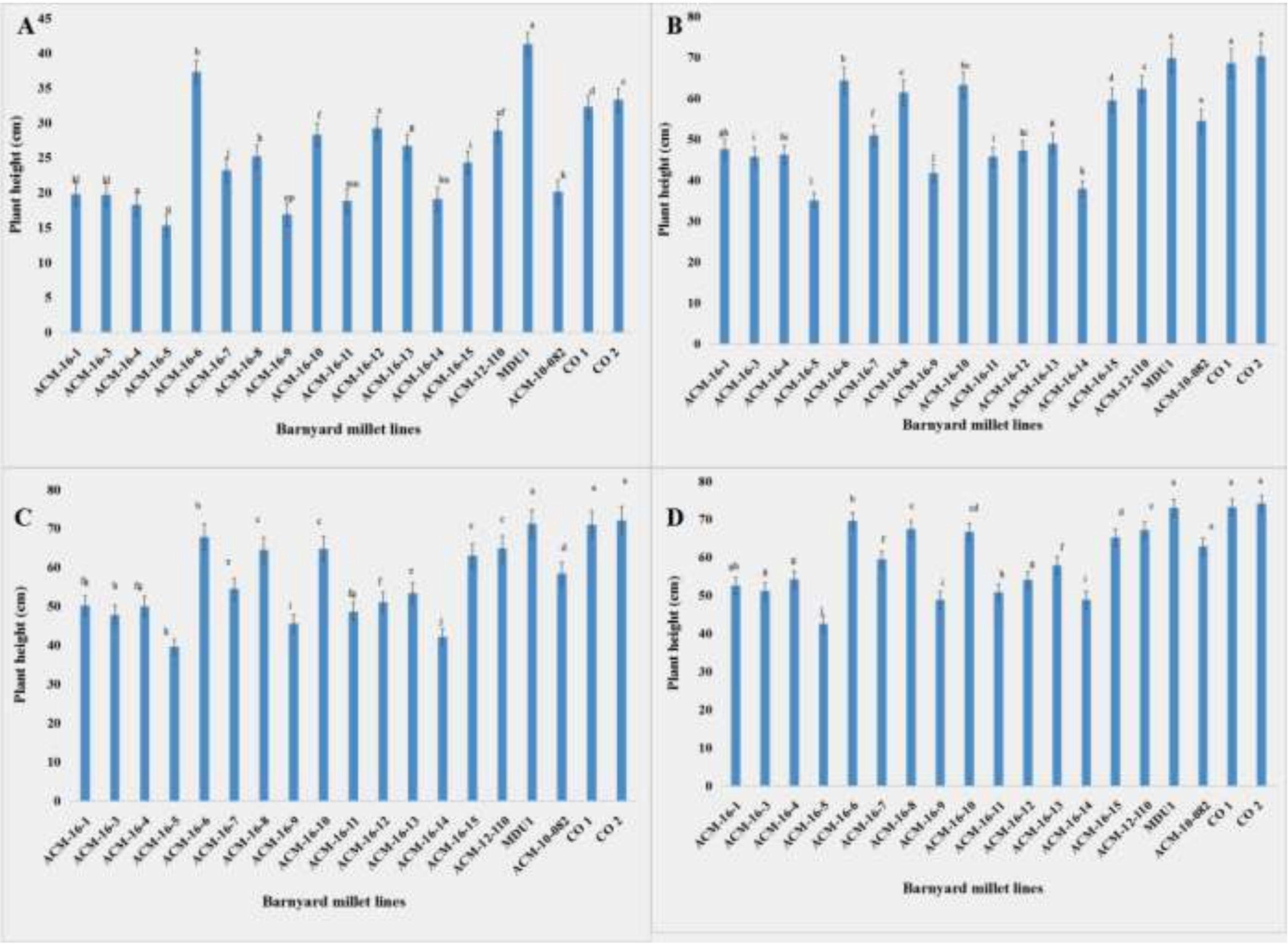

Fig. 1. Plant height of barnyard millet lines at different growth stages. A) Seedling B) Vegetative C) Flowering D) Maturity. Data represent mean ± SE and different letters denote significant differences between the lines according to completely randomized design analysis at P<0.05, n-3.

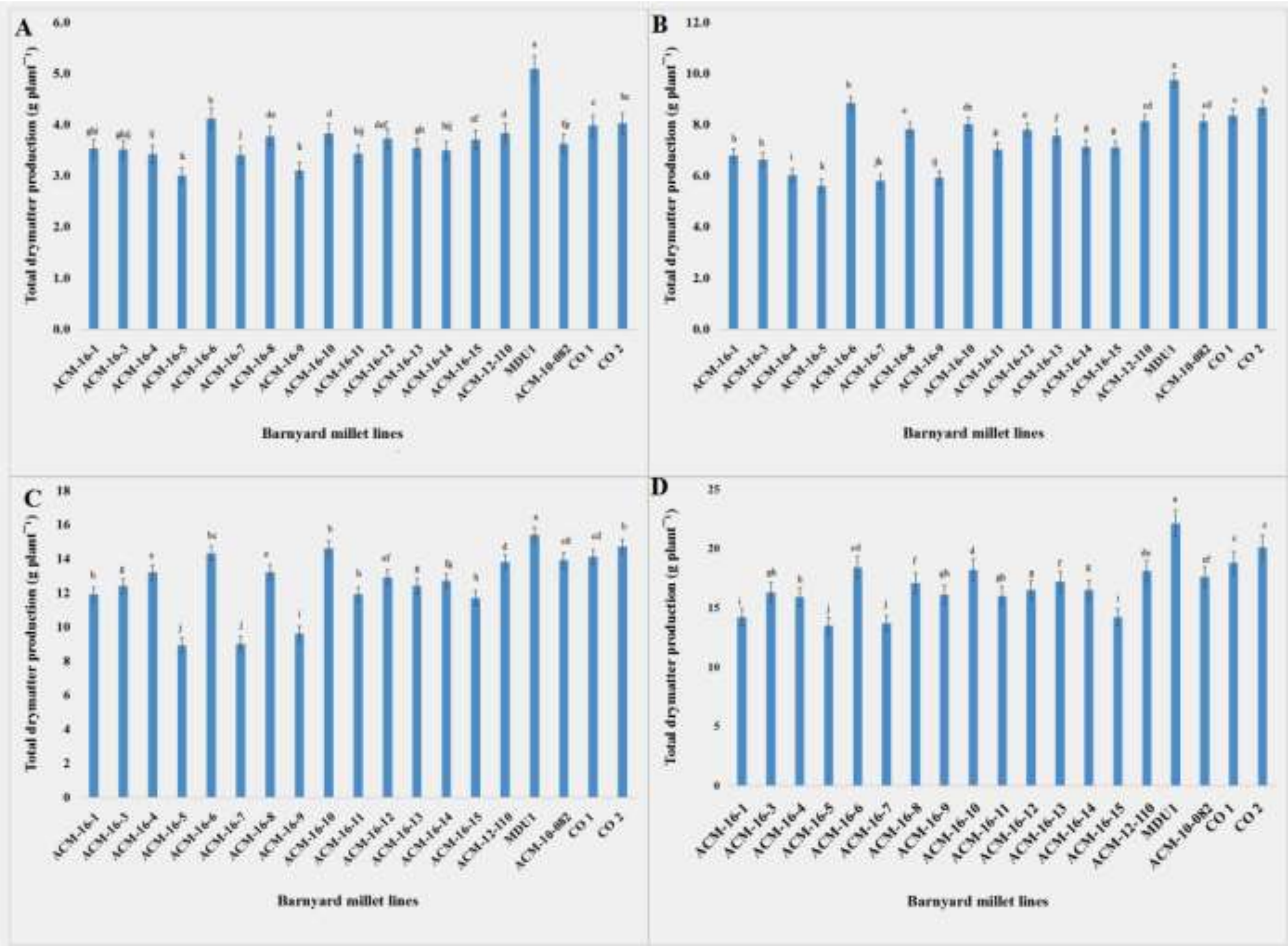

Fig. 2. Total dry matter production of barnyard millet accessions at different growth stages. A) Seedling B) Vegetative C) Flowering D) Maturity. Data represent mean ± SE and different letters denote significant differences between the lines according to completely randomized design analysis at P<0.05, n-3.

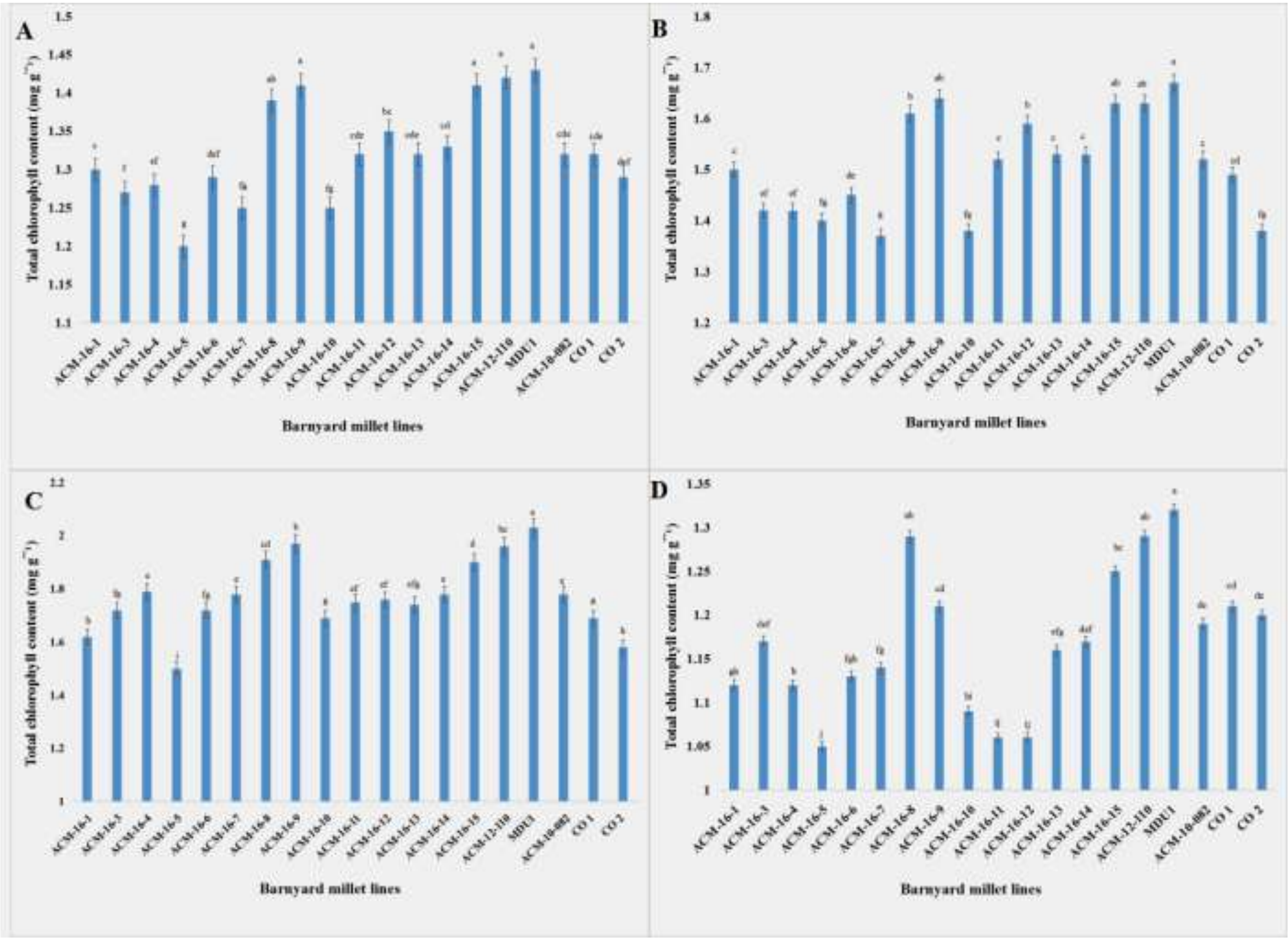

Fig 3. Total chlorophyll content of barnyard millet accessions at different growth stages. A) Seedling B) Vegetative C) Flowering D) Maturity. Data represent mean ± SE and different letters denote significant differences between the lines according to completely randomized design analysis at P<0.05, n-3.

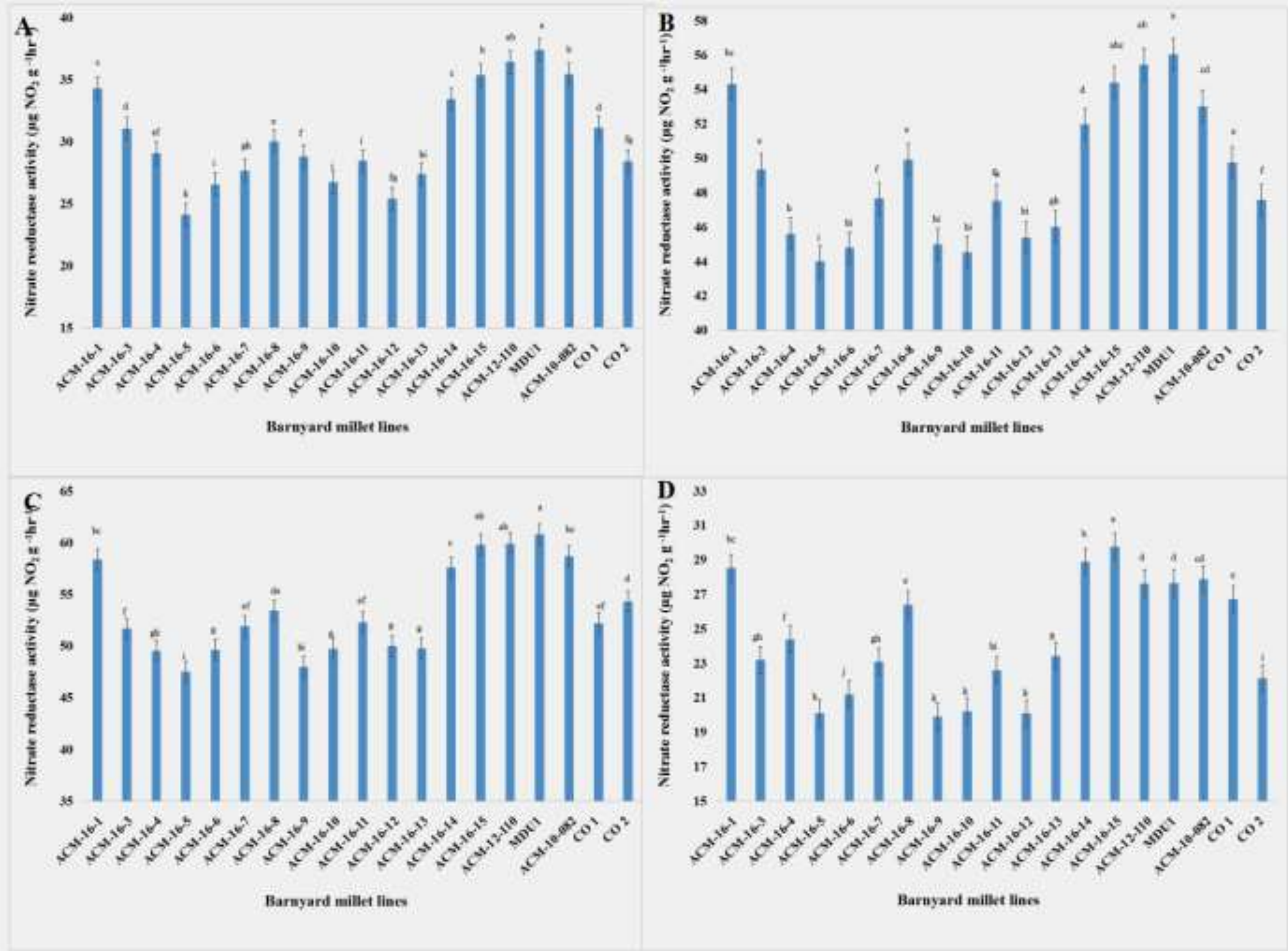

Fig 4. Nitrate reductase activity of barnyard millet accessions at different growth stage. A) Seedling B) Vegetative C) Flowering D) Maturity. Data represent mean ± SE and different letters denote significant differences between the lines according to completely randomized design analysis at $P<0.05$, n-3.

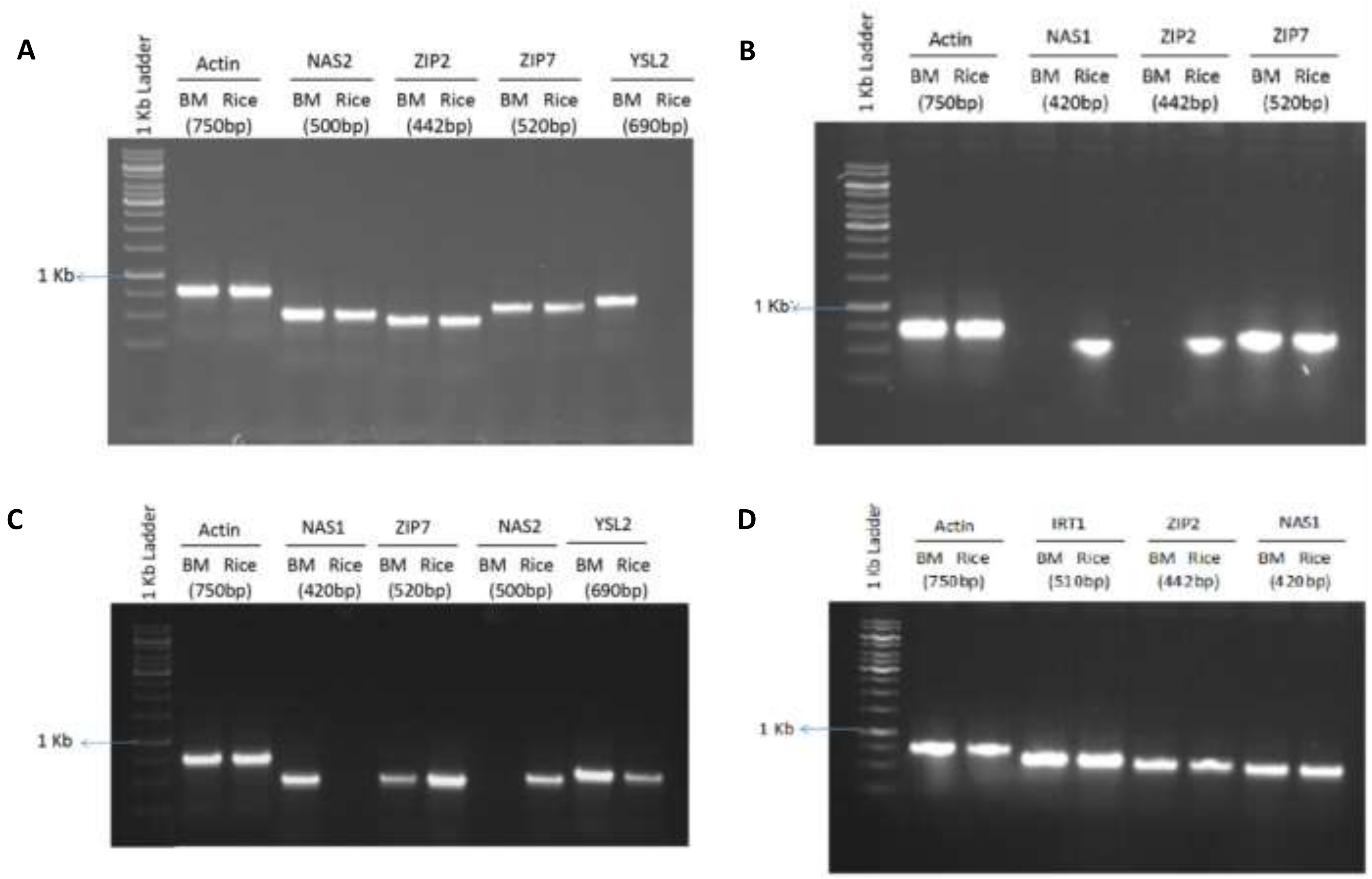

Fig. 5A-D. Expression profiling of metal homeostasis genes in barnyard millet (MDU1) and rice ASD16) at seedling and vegetative stages. A) Leaf tissue of MDU1 at seedling stage B) Root tissue of MDU1 at seedling stage C) Leaf tissue of MDU1 at vegetative stage B) Root tissue of MDU1 at vegetative stage.

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