Indian J. Applied & Pure Bio. Vol. 39(2), 1117-1126 (2024). **ISSN: 0970-2091**
A Web of Science Journal

ICPOES Based seed micronutrient content analysis as Tool to identify Allelic Sergeants' in a Stable RIL mapping population of Foxtail millet (*Setaria italica* L.)

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Abstract

Foxtail millet (*Setaria italica* L.) is a small grained C4 Panicoid cereal and a family member of Poaceae, with a small genome size of about ~515 MB. It serves as staple food in dominant arid and semi-arid regions of Asia and Africa. Being a fact that millets are nutritionally superior, rapid urbanization and green revolution has contributed for substantial shrinkage of millet cultivable area. Reduced freshwater, fertile lands and unpredicted environmental factors has led to the comeback of the millets as an answer to global Nutri-food security challenges. Based on earlier work from our lab, genetically distinct genotypes for seed micronutrients and yield related traits were used as parental lines for stable RIL mapping development. Testing the hypothesis of segregating alleles in the population, randomly selected RIL's along with their parental lines were subjected to ICP-OES analysis. High genetic variability for the seed micronutrient content with respect to iron, zinc, copper and manganese has been observed in the RIL's. Descriptive statistics and frequency distribution reveals the high micronutrient contents in developed RIL population when compared with their parental lines. The results are evident of the segregating genetic determinants controlling seed micronutrient content that can be used for breeding foxtail millet for enhanced micronutrient content.

Key words : ICPOES, micronutrient Allelic sergeants, RIL mapping. Foxtail millet (*Setaria italica* L.).

Since the inception of civilization, some grains have been farmed and produced for both human food and animal feed. Among the most extensively consumed, produced, and farmed cereal grains (whole or fractionated) are now essential components of an individual's regular diet[1]. The majority of people on the globe primarily rely on cereal-based diets, yet these diets are deficient in important micronutrients, which puts consumers at serious risk of nutritional insecurity[12]. Because of their high protein, mineral, vitamin, and antioxidant content, millets are referred to be "nutritious millets" or "Nutri cereals," since they offer greater nutrition compared to non-millet cereals. The government recognised the importance of millets by declaring 2023 as the International Year of Millets. Millets are ancient crops with a long history of cultivation, and they have been vital to the survival of various cultures across the world. Millets generally provide extremely nutritious, non-glutinous, and non-acid producing diets. Although both foxtail millet and proso millet include both glutenous and non-glutenous kinds of grains[10]. In addition to their high micro- and macro-nutrient contents, millets also encompass enhanced levels of low GI non-starchy polysaccharides and dietary fibre. The micronutrients iron and zinc content of the grains were also found to be the highest, suitable for expectant mothers and children who suffer with anaemia, in most of under-developed Nations like India[9]. Foxtail millets are multifunctional, very old and valuable crop that serve the main diet in China[15]. Foxtail millet grains, which are gluten-free carbohydrate, high protein containing small grain loaded with high fibre, calcium, zinc, iron, vitamin, and lipid content. Millets have a calming, alkaline impact on the digestive tract that helps the body maintain a proper pH balance, which is crucial for immunity[12]. Because millets lack of gluten, they are considered non-allergenic[2]. From a consumer perspective, it is important to emphasise that although millets are more nutritious than rice and wheat, their use is not as prominent, rather than wheat and rice, millets ought to be the chosen food. However, improving its yield and nutritional quality remains a priority. Being the crops of shorter life-cycle, millets therefore are ideal staple crop for growing population. It has twice the content of nutritional content as compared to rice[11]. Biofortification of edible crops as method to combat malnutrition has been addressed by many workers[7].

The main objective of this study was to establish segregating alleles in the developed stable RIL mapping population compared to their parental lines. Towards the goal, randomly selected ten RIL sergeants' along with their parental lines were studied using ICPOES and the data was subjected to descriptive, clustering and principal component analysis of nutrient quality characters. The results were evident that a substantial variation among the mapping population compared to their parental lines that have the breeding value to answer the nutritional quality in foxtail millet.

Selection of parental lines :

High-yielding landraces and released cultivars with superior nutritional profiles were selected as parental lines viz., Black, a landrace and Srilakshmi, a released cultivar. These genotypes possessed desirable agronomic traits, including increased nutritional content, serving as the foundation for the development of the mapping population (Kola *et al.,* 2020; Ramesh *et al.,* 2023)

Population Development :

Systematic crosses were performed to generate a genetically stable mapping population, viz., BLACK (Landrace) x SRILAKSHMI (Released cultivar) to enhance the yield and nutritional quality. Based on the Morpho-Phenological traits, true F_1's were identified (Sameena *et al.,* reported somewhere else) and advanced to generate stable segregating RIL population. Testing the hypothesis of segregating alleles in the population for seed nutritional content, randomly selected ten RIL's from the Black*Sri Lakshmi population along with their parental lines were subjected to ICP-OES analysis (Table-1).

Plant seed material preparation :

The fully matured grains of RIL's derived from Black *Sri Lakshmi via systematic crossings were planted in a fully random blocked design in three replicates of each sample in the well-prepared seedbeds of natural field soil. Following the standard agricultural procedures, the lines were cultivated in net house at Yogi Vemana University in Kadapa, Andhra Pradesh, under natural environmental conditions (30±1ºC/37±1ºC with relative humidity varying from 50–80%). After maturing, panicles were harvested and seeds were separated, sundried and stored in cool dry place until further use. A cleansed mortar and pestle were used to dehusk and crush the seeds into flours. To eliminate any moisture content, the flours were dried in a hot air oven at 55°C for four to five hours. Nutrient analyses were performed on dehydrated flours to determine the presence of micronutrients

Preparation of sample for ICP-OES Analysis:

A clean, acid-prewashed polypropylene tube was filled with 500 mg of dehydrated flour from each of the ten randomly chosen recombinant inbred lines and their parents in three replications. In a closed vessel microwave digestion system, the samples were broken down using 2 ml of HNO_3 (65%V/V) and 0.5 ml of H_2O_2 (30% H_2O_2), and the vessel was left to remain at room temperature for the overnight (cold digestion). The sample-acid mix was heated for 30 minutes at 125ºC to facilitate digestion; the vessel is sealed with a screw lid. After allowing the samples to cool, distilled water was added to make up to 10 ml. To ensure adequate mixing, the mixture was placed in an orbital shaker for one minute. Whatman No. 1 filter paper was then used to filter the samples. For micronutrient analysis, the supernatant was analysed via ICP-OES (ICPOES HD Prodigy –Lemans). The following were the ICP-OES working conditions: 1.1 kw of RF power, 18 L/min of coolant flow, 0.0 L/m of auxiliary flow, Table-1 : 3 Landraces,1 Released cultivar and 10 RIL's of Foxtail millet

S.No.	Name of foxtail millets	Variety Type	Description
1	Black	Landrace	Parents
2	Srilakshmi	Released cultivar	Parents
3	B*SL1	RIL	RIL
4	B*SL2	RIL	RIL
5	B*SL3	RIL	RIL
6	B*SL4	RIL	RIL
7	B*SL5	RIL	RIL
8	B*SL6	RIL	RIL
9	B*SL7	RIL	RIL
10	B*SL8	RIL	RIL
11	B*SL9	RIL	RIL
12	B*SL10	RIL	RIL

Hildebrand type Nebulizer, 34 PSI of Nebulizer pressure, 1.4 ml/min of sample uptake, and cyclonic Spray Chamber Dual vision torch with a pure nitrogen gas flow of 0.7 litres per minute; Wash time is 40 seconds, axial time is 10 seconds, and radial time is 5 seconds. Every sample was examined three times. The instrument's built-in software read the data. Table-2 describes the elemental parameters of the micronutrients that were examined using ICP-OES analysis.

Statistical analysis :

The estimation of mean, maximum, minimum, variance, standard deviation, kurtosis, skewness and percent of coefficient variation of the micronutrient data were worked out by adopting the standard method[6] XLSTAT 2019.3.1.60379 software for principal component analysis (PCA) followed by the "R" program for multivariate hierarchical cluster analysis.

Table-2. Elemental parameters of ICPOES analysis

Element	View	Wave length
Copper	Axial	327.393
Zinc	Axial	213.856
Manganese	Axial	257.610
Iron	Axial	259.940

Descriptive statistics :

This process aimed to capture the genetic diversity necessary for a comprehensive understanding of nutritional and yield-related traits. Seed micronutrient content viz., Fe, Mn, Cu and Zn has been studied in randomly selected ten lines (subset) out of 180 stable RIL's along with parental lines. The values of the micronutrient data were subjected to statistical analysis in order to study the descriptive statistics like maximum, minimum, mean, Standard Deviation, variance, skewness, kurtosis and coefficient of variation (%) (Table 3 a & b). Both the parents show the highest mean in Zinc (Zn) with 3.65mg/100g whereas RIL's shows high in Iron (Fe) with 2.751mg/100g low in 0.355mg/100g in Manganese (Mn) in parents and 0.356mg/100g in copper (Cu) in RIL's. The Elements like copper, zinc and iron were found positively skewed while Manganese shows negatively skewed in RIL's. Maximum kurtosis was observed in Zinc (Zn) while the minimum kurtosis was observed in Iron. The coefficient of variation ranges between 0.102 to 1.16% in RIL's. Total seed micronutrients in a F_2 population was found to be segregating with varied concentrations, compared to their parental types[9]. Recombinant inbred lines were characterized into four different classes based on the range observed for each element. Most of RIL's were high in Iron and manganese low in copper and intermediate in zinc when compared to parents. The same pattern of the allelic segregants were observed in the present study compared to their parental types as illustrated in histograms (Fig. 1).

Cluster analysis :

Hierarchical cluster analysis was performed to identify the concentration of micronutrient variability in segregating lines compared to their parents using r programme software package. The dendrogram thus generated in the present study, revealed that

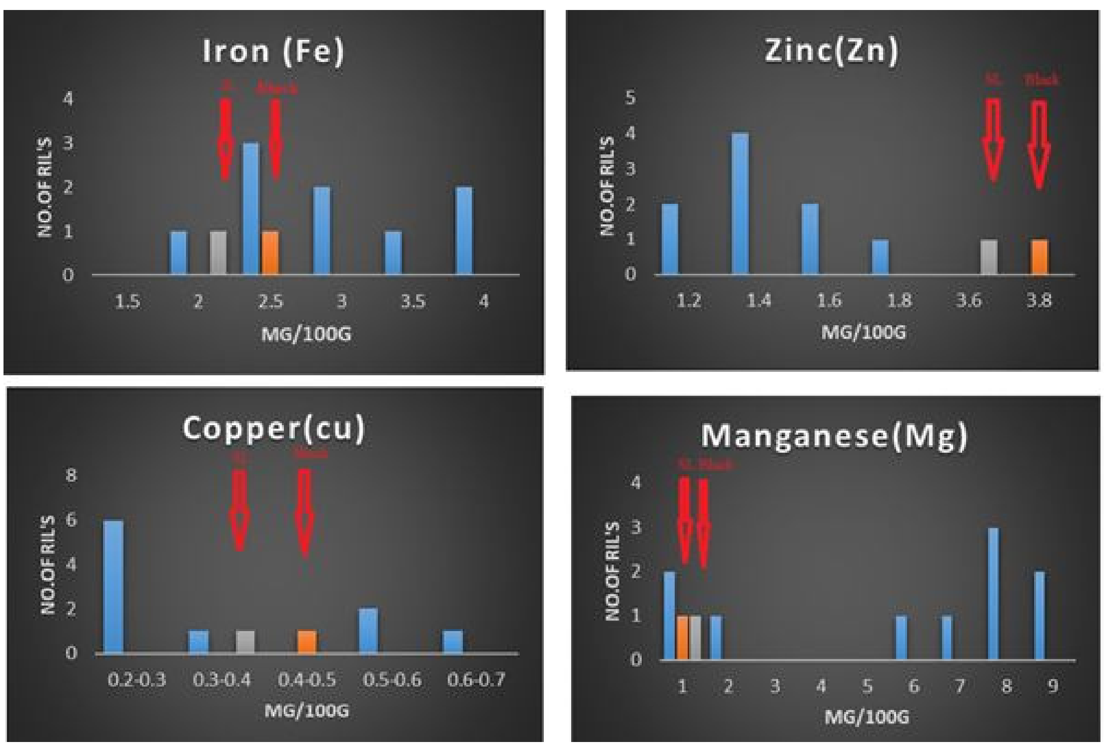

Fig. 1. Frequency Distribution of micro nutrient elements in RIL's along with their Parents

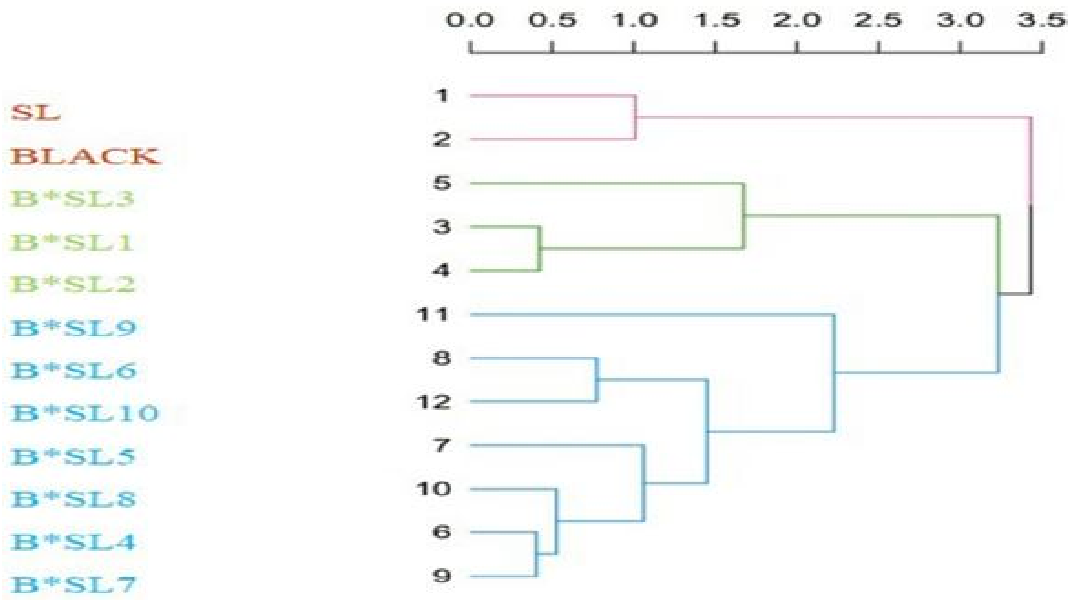

Fig. 2. Hierarchical cluster dendrogram showing clusters in parents and RIL's of Foxtail millet under various macronutrient concentration

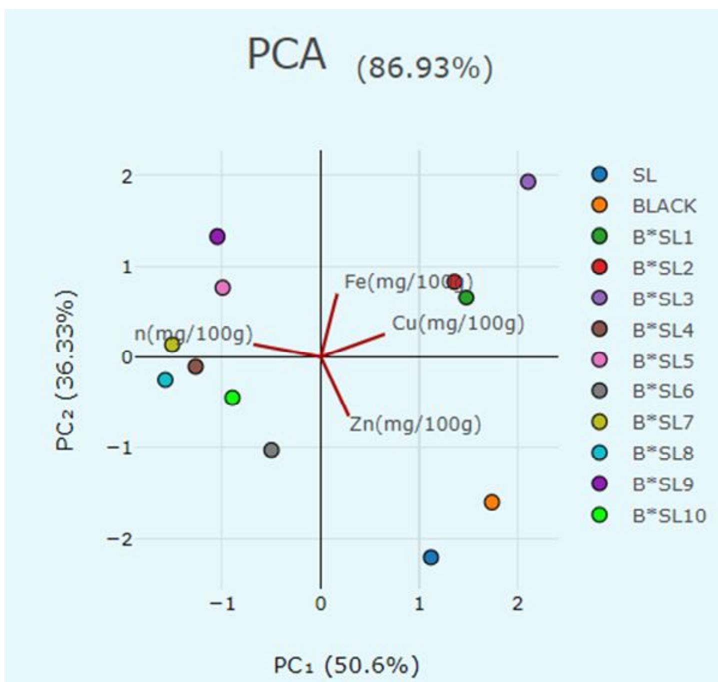

Fig. 3. Bi plot formation on basis of PC1 and PC2 values

Table-3(a). Mean values of different Micronutrient contributing elements in foxtail millet Parents

Particulars	Max.	Min.	Mean	S.D	Varia-nce	Skew-ness	Kur-tosis	CV %
Iron (Fe)	2.5	2	2.25	0.353	0.125	-	-	3.176
Zinc (Zn)	3.7	3.6	3.65	0.070	0.005	-	-	0.635
Copper (Cu)	0.44	0.35	0.395	0.063	0.004	-	-	0.571
Manganese (Mn)	0.38	0.33	0.355	0.035	0.001	-	-	0.317

Table-3(b). Mean values of different Micronutrient contributing elements in foxtail millet RIL's

Particulars	Max.	Min.	Mean	S.D	Varia-nce	Skew-ness	Kur-tosis	CV %
Iron (Fe)	3.9	1.67	2.751	0.710	0.504	0.392	-0.341	0.508
Zinc (Zn)	1.7	1.1	1.39	0.166	0.027	0.014	0.784	0.118
Copper (Cu)	0.61	0.21	0.356	0.143	0.020	0.966	-0.831	0.102
Manganese (Mn)	4.5	0.38	2.707	1.632	2.666	-0.628	-1.470	1.168

Table-4. Principal component analysis of four mineral elements in foxtail millet genotypes showing Eigen values and their percentage contribution to the total variation

Para-meters	Cu(mg/ 100g)	Mn(mg/ 100g)	Fe(mg/ 100g)	Zn(mg/ 100g)	Eigen value	% of Variance	Cumul-ative (%)
PC_1	-3.238	2.708	1.436	-0.907	2.024	50.601	50.601
PC_2	-1.095	-2.117	2.177	1.036	1.453	36.326	86.927
PC_3	-0.726	0.147	-0.949	1.528	0.500	12.516	99.445
PC_4	-8.882	4.441	1.11	-1.943	0.022	0.555	100

the elucidean distance ranging from 0-3.5 which reveals the high genetic variability in between parents and RIL's. The dendrogram were grouped it into 3 main shown in figure 2. distinct clusters. cluster 1 was consists of only two lines (Parents) Black and Srilakhsmi this cluster was characterized by high content of zinc (Zn) followed by 3 RIL's with high content of copper in cluster 2 and seven in cluster 3 with high content of copper. The clustering pattern was represented using the dendrogram (Fig. 2). Numerous cluster analysis studies have been conducted on the micronutrients of RILs of cereals such as Kodomillet[14], finger millet[3], little millet[14], and pearl millet[8].

Table-5. The scores of the four rotated principal components

Species	PC_1	PC_2	PC_3	PC_4
SL	1.119	-2.209	0.515	-0.179
BLACK	1.738	-1.599	0.890	0.171
B*SL 1	1.478	0.652	-0.564	-0.142
B*SL 2	1.358	0.827	-0.902	-0.006
B*SL 3	2.105	1.934	0.178	0.098
B*SL 4	-1.270	-0.107	-0.047	0.096
B*SL 5	-0.995	0.764	0.279	-0.139
B*SL 6	-0.500	-1.025	-0.951	0.103
B*SL 7	-1.508	0.138	0.057	0.283
B*SL 8	-1.578	-0.253	-0.133	-0.155
B*SL 9	-1.049	1.327	1.351	-0.072
B*SL 10	-0.896	-0.450	-0.663	-0.060

Principal component analysis :

In the present study correlation was observed in mean values of four micronutrients of the parents and RIL's. The principal component analysis grouped the Parents and RIL's based on the variables of Iron, Zinc, Copper, and Manganese. PCA demonstrated that out of four; the first two principle components elucidated superiority of total variation. principal component analysis showed the total variation of first two principal components is 86.93% with the >3 eigen value these first two interaction principal components were significant for both the Parents and RIL's with PC I on X-axis and PC II on Y- axis. Out of four PC's, PC I and PC II have eigenvalues of 2.024, and 1.4531 respectively. PC III had the eigenvalue 0.5007and PC IV had the eigen value of 0.022(Table4). The PC I contributed maximum (50.60%) followed by PC II (36.32 %), PC III (12.51%). PC IV (0.5555 %) In the present study the PC I had the highest positive loading for manganese (2.708) and higher negative loading for copper (-3.2385). In PC II, Iron (2.177), zinc (1.036) has positive loadings and manganese (-2.1777) and copper (-1.095) having a negative loading. PC III attributed to Zinc (1.528) and copper (-0.726) with positive and negative loadings respectively. The PC IV consists of variables regards to Manganese (4.441) with positive loadings and copper (-8.882) negative loadings (Table-4).

The principal component output of the foxtail millet parental lines and segregating population was as represented in Table 5. Based on the scores, the PC1, PC II, PC III and PC IV component explains that all the micronutrient concentrations are higher in RIL's when compared with parents. PCI and PC II values of the present principal component score plot has clearly grouped the parents and RIL's into four quadrants depending on the concentrations of four micronutrient elements. Out of four quadrants based on the compositions of micronutrients, parents and RIL's were scattered into four quadrants in PC analysis. From the results it can be concluded that RIL's shows wide range in between them and also when compared with parental lines like iron and copper shows positive in RIL's

This research provides a extensive comparison of the micronutrient content of foxtail millet between parents and RILs using multivariate statistical analysis of ICP-OES data. The generated mapping population provides a platform for additional study into the genetic foundation of nutritional aspects in foxtail millet, which is a valuable resource for both breeders and researchers. The significance of landraces that are underutilised early in breeding programmes was revealed by our findings. This work contributes to the global food and nutrition security by addressing the rising demand for nutritionally improved crops and laying the foundation for focused breeding efforts aimed at enhancing the overall nutritional quality of foxtail millet. This study reveals the wide range of variation for all the micronutrients except zinc in RIL's when compared with their parental lines and shows positive corelations. Iron, calcium and Manganese content in Black *Srilakshmi would be rewarding in selection programme for maximum improvement in yield of foxtail millet. The breeders without compromising on grain yield could develop the maximum micronutrient rich varieties. Developing a mapping population from landraces with improved nutrition is a determined tactic to identify the genes affecting foxtail millet yield-related characteristics. This research contributes to the overarching goal of harnessing the genetic potential of foxtail millet for improved human nutrition and global food security.

This work was supported by the Department of Science and Technology (DST)-INSPIRE, New Delhi as part of a Ph. D degree scholarship to SS (IF170752) and KSSR (IF220369). AK thanks for the JRF (No. CRG/2018/003280 dated 30th May 2019) from DST-, Science and Engineering Research Board (SERB), New Delhi, India. ACS and PCOR in YVU, acknowledge the partial utilization of the financial support for consumables (No. CRG/2018/003280 dated 30 May 2019) from Department of Science and Technology, Science and Engineering Research Board (DST-SERB), New Delhi, India.

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