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Metabolomic characterization of barnyard millet (*Echinochloa frumentacea* L.) involved in different stages of spike development

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Abstract
The nutritional value of millet is dependent upon its metabolic profile, including the types and amounts of natural phenolic compounds present, which warrant evaluation in order to determine the best genotypes for human consumption. Barnyard millet (*Echinochloa frumentacea* L.) could be a good source of iron for vegetarians. In the present study, Metabolome profiling of barnyard millet genotypes containing high Fe and low Fe content in five spike development stage was performed using GC-MS platform and results observed highest percentage of sugar and sugar alcohol (34%) followed by organic acid (26%), Amino acids, sterol, other compound (23%) and fatty acid (17%) in spike development stages of Low Fe Vs High Fe genotypes. Heat map revealed that during spike emergence the metabolic compounds like d-Ribose, D-Fructose, D-Glucose, Galactose, D-Turanose, Glucopyranose, D-Mannitol, Hexadecanoic acid, Docosanoic acid, alpha. - Glycerophosphoric acid and beta.-Sitosterol were found high in high Fe genotype in comparison to Low Fe genotype. The hierarchical cluster analysis, revealed that high Fe five spike development stages shares close metabolite pool to each other and in low Fe four spike development stages also shares similarly metabolite pool to each other except spike emergence stage of Low Fe genotype. PCA showed that the expression patterns of the five developmental stages differed significantly.

Keywords: Barnyard millet, GC- MS, metabolites compound, heat map, hierarchical clustering analysis, principal component analysis
Abbreviations: dH_2O Distilled water, GC-MS Gas chromatography-mass spectrometry, IIMR Indian Institute of Millets Res, H Fe High Iron, L Fe Low iron, S1 Spike emergence, S2 Pre-Pollination, S3 Pollination, S4 Milking, S5 Seed maturation, PLS-DA Partial least squares-discriminant analysis

Introduction
Barnyard millet is a multi-purpose crop which is cultivated for food and fodder. Barnyard millet grains are a rich source of dietary fiber, iron, zinc, calcium, protein, carbohydrate, magnesium, fat, vitamins, and some essential amino acids and, most notably, contains more micronutrients (iron and zinc) than other major cereals. Barnyard millet could be a good source of iron for vegetarians. Some of its varieties have high amount of iron which is the richest amongst all millets and cereal grains.
The nutritional potential of millets is limited by the presence of phytates, phenols and tannins. Kulkarni and co-workers assessed the tannin content of five minor millets *viz.*, proso, kodo, Italian, little and barnyard millet and recorded lowest level in barnyard millet (102.96 mg). It has been shown that dehulling of the seeds reduces phytate and tannin levels (Kulkarni *et al.*, 1992) [1]. Thus, for the health-conscious genera of the present world, minor millet especially barnyard millet is one more addition to the existing list of healthy foods, owing to its nutritional superiority (Padulosi *et al*, 2009) [2].
Metabolomics aims at determining a sample's metabolites profile and hence provides a straight functional statement of an organism's physiological condition. The nutritional quality of millet grains is usually equivalent or superior to that of other cereals containing high amounts of minerals, essential amino acids, carbohydrates, and vitamins (Lorenz and Hinze, 2002; Taira, 2002; Yang *et al.*, 2013) [2, 3, 4]. However, its advantage has not been mirrored to the same extent in dissecting metabolic pathways because of the relative lack of knowledge of both primary and secondary metabolism in this species (Suma and Urooj, 2012) [6].
A combination of gas chromatography and mass spectrometry (GC-MS) allows for the identification and robust quantification of several hundred metabolites within a single extract. The primary metabolite profile is closely related to the organism's phenotype and includes important nutritional characteristics (Hoekenga, 2008; Kok *et al.*, 2008) [7, 8].

In this study, Metabolic profiling in whole millet grains from high and low Fe content of two genotypes using GC-MS coupled with chemo metrics was applied to determine the phenotypic variation and to analyze relationships between their contents. Therefore, the nutritional value of millet is dependent upon its metabolic profile, including the types and amounts of natural phenolic compounds present, which warrant evaluation in order to determine the best genotypes for human consumption. Plant identification and differentiation at the species, population and individual genotype levels is of major importance for plant scientists and breeders. It has already been employed to direct breeding strategies for improving and optimizing the balance of food components. Consumers are aware of the need for a constant supply of phytochemical-containing foods for antioxidant support and disease prevention (Kim *et al.*, 2013) [9].

The Metabolomic profiling of barnyard millet will provide complete picture of available essential amino acids, minerals, vitamins and bioavailability of nutrients. It will also provide information on anti-nutritional components which can be improved by the use of novel method created from the information generated.

However, to the best of our knowledge, Metabolomic profiling in various genotypes of barnyard millet have not been reported. There is no single report of metabolite profiling of barnyard millet in Fe content of spike developing stages. In the present study metabolome profiling of barnyard millet genotypes containing Low Fe and High Fe content was carried by GC-MS platform.

Materials and Methods

Sample Collection

Thirty genotypes of barnyard millet were procured from IIMR, Hyderabad. Samples were collected based on elemental analysis of iron content of barnyard millet genotypes. Spike development stages (Figure 1) samples were collected from Kharif season: 2018-19 (August-October). For GC-MS analysis samples were kept in aluminum foil in -80ºC for longer period of time.

Extraction and derivatization of metabolites

Metabolomic (whole metabolomic) study was performed using GC–MS. Metabolites were extracted as described by Velledor *et al.* (2014) [10] with minor modifications. Spike tissues (200 mg) were homogenized with pre-chilled mortar-pestle in 3 ml of 100% HPLC grade methanol (pre cooled at −20 °C). The mixture was shaken for 10 min at 70 °C in a water-bath at 950 rpm and centrifuged for 10 min at 11,000 g. The supernatant was transferred to a SchottGL14 glass vial and 1.5 ml of chloroform (−20 °C) was added. After that 3.0 ml of dH2O (4 °C) was added and vortexed for 10s. Again, the mixture was centrifuged for 15 min at 2200g and the upper phase (polar) and lower phase (nonpolar) phase were transferred into a separate test tube. Bothpolar and nonpolar phase were dried in a nitrogen stream. Extracted metabolites were derivative as described by Sanimah *et al.* (2013) [11] with minor modifications. The dried extracts were re-dissolved in 50 µl of pyridine and sonicated for 10 min. Then, 100 µl of methoxyamine HCL (20 mg ml−1in pyridine) was added and vortexed for 30 s. The mixtures were then sonicated again for 5 min and incubated with constant agitation for 90 min at 37 °C. The trimethylsilylation (TMS) step was performed by adding 250 µl N-Methyl-N- (trimethylsilyl) trifluoro acetamide (MSTFA) to the extracts and vortex for 30 s. Mixtures were incubated for 1 h at 37 °C for derivatization.

Metabolomic analysis by GC-MS

For GC–MS analysis, 1 µl of derivative extract was injected into a DB-17MS capillary (30 m × 0.25 mm). The inlet temperature was set at 280 °C. After a solvent delay for 5 min, initial GC oven temperature was set at 100 °C; after injection for 1 min, the GC oven temperature was raised to 290 °C.

The injection temperature was set to 280 °C and ion source temperature was 230 °C. Helium was used as the carrier gas with a constant flow rate set at 1 ml/min. The measurement was performed with electron impact ionization (70 eV) in the full scan mode (m/z from 50 to 700). Metabolites were putatively identified by matching their mass spectra to spectra in NIST 14 library (National Institute of Standards and Technology, Gaithersburg, MD, USA). Pre-processing of total ion chromatograms (TIC) such as baseline correction, alignment, peak picking, and integration were performed using the ACD/Spec Manager v.12.00 (Advanced Chemistry Development, Inc., ACD/Labs, and Toronto, Canada). CSV comma delimited files were created for data analysis.

Data processing and statistical analysis

Data processing and statistical analysis of metabolites was carried out using Metabo Analyst 4.0 (Chong *et al.*, 2019) [12], an online statistical package and the data were normalized and processed. Data were normalized with Pareto scaling for metabolomic analysis. Heat Map, Hierarchical cluster analysis and Principle Component Analysis (PCA) was performed using same package.

The relative concentrations (g/DW) of different metabolites for High Fe and Low Fe five spike development stages treatment were formatted as comma separated values (.cvs) files. The cvs file was uploaded to the Metabo Analyst 4.0 for successive analysis. To improve data quality for performing downstream statistical analysis, the data quality was checked and normalized by sum, log transformation and auto scaling.

Results

The current study carried out to understand metabolic alteration in different parts of the plant at the spike development stage that could provide a more precise indication of developmental changes in plants. In the present study a total of 35 metabolites of known structure comprising sugars, sugar alcohols, fatty acids, amino acids, organic acids, sterols and other were identified at Low and High Fe spike development stages as determined from chromatogram. Using the NIST library, metabolites were found highest amount of sugar and sugar alcohol (34%) followed by organic acid (26%). Amino acids, sterol and other were found (23%) and fatty acid (17%) in spike development stages of Low Fe and High Fe content genotypes (Figure 2). Total number of metabolites produced in each stage given in supplementary table (Table 1). Khan *et al* (2017) [13] reported GC-MS chromatograms profile of the methanolic extract of pearl millet genotypes showed the number of phytochemical constituents and identified different chemical classes of unsaturated fatty acid, aldehydes, organic acids, sterol and amino acids compounds in all genotypes of pearl millet. Similarly, Romina *et al.* (2009) [14] studied metabolic profiling and analysis of volatile composition of durum wheat semolina

and pasta and they found 34 metabolites compounds including amino acids, sugars, organic acids, fatty acid and sugar-alcohols.

Heat map of metabolites (Table 2 and Figure 3) showed that common metabolites compound in High Fe spike five development stages were found higher accumulation than in Low Fe spike development stages. High Fe Spike emergence and Low Fe spike emergence stage found similar 12 compounds of metabolite but in High Fe Spike emergence increase accumulation of metabolite compounds like d-Ribose, D-Fructose, D-Glucose, Galactose, D-Turanose, Glucopyranose, D-Mannitol, Hexadecanoic acid, Docosanoic acid, alpha.-Glycerophosphoric acid and beta.-Sitosterol than Low Fe Spike emergence stage. In both pre pollination stage of Low Fe and High Fe similar 8 compounds of metabolite observed in which High Fe pre pollination stage metabolite compounds like Putrescine, L-Proline and Hexadeconoic acid were showed higher accumulation than Low Fe pre pollination stage. Pollination, Milking and Seed maturation stages of High Fe genotype observed more metabolite compounds than Low Fe genotype stages. In pollination stage of high Fe increase accumulation of metabolite compounds like Butanedioic acid, Malic acid, 2,3,4-Trihydroxybutyric acid, Glutamine, L-Proline, Pentitol, D-Fructose, D-Glucose, Galactose, 1,2,3-Propanetricarboxylic acid, D-Mannitol, Hexadecanoic acid and Stigmasterol than low Fe pollination stage. High Fe milking stage showed 23 compounds of metabolite and 13 compounds in Low Fe milking stage. In which compound like 2,3,4-Trihydroxybutyric acid, d-Ribose, Ribonic acid, Pentanedioic acid, Pentitol, D-Mannitol, alpha.-Glycerophosphoric acid, Eicosanoic acid, Stigmasterol and beta.-Sitosterol observed higher accumulation in HighFe_milking stage and compounds like Malic acid, Glutamine, 1,2,3-Propanetricarboxylic acid, Tetradecanoic acid, D-Mannose and Talose observed higher in Low Fe milking stage. Compounds of metabolite found in seed maturation stage of High Fe and in Low Fe seed maturation stage were 17 and 10 respectively. 14 metabolite compounds showed higher accumulation in High Fe seed maturation stage like Butanedioic acid, 2,3,4-Trihydroxybutyric acid, Glutamine, L-Proline, Tetradecanoic acid, Hexadecanoic acid, alpha.-Glycerophosphoric acid, 9,12-Octadecadienoic acid, D-Mannose, D-Glucuronic acid, Eicosanoic acid, Docosanoic acid, D-Glucopyranose, Stigmasterol and beta.-Sitosterol and 3 metabolite compounds showed higher in Low Fe seed maturation stage like Malic acid, Glucaric acid and D-Xylopyranose. In genotypes High Fe found constant express metabolite compounds were Butanedioic acid, 2, 3, 4-Trihydroxybutyric acid, Glutamine, L-Proline, Ribonic acid, alpha.-Glycerophosphoric acid, D-Mannose, D-Glucuronic acid and Stigmasterol in pollination, milking and seed maturation stage.

Based on dendrogram analysis (Figure 4) metabolites of spike developmental stages of High Fe and Low Fe genotypes were scattered into two main clusters. Cluster 1 represents clusters among all sample from that stage seed maturation of Low Fe genotype showed from the all the sample that may be have different metabolite pool differentially expressed during the spike development. In cluster 2 further divided into 3 and 4. In cluster 3 High Fe pollination stage showed different metabolite pool from High Fe pre pollination, High Fe spike emergence and Low Fe spike emergence stage. In cluster 3 further divided into 5 and 6. In cluster 5 showed different metabolite pool in High Fe pre pollination stage from High Fe spike emergence and Low Fe spike emergence stage and results said that the High Fe pre pollination stage close to High Fe pollination stage. In cluster 6 found similar metabolite pool in both High Fe spike emergence and Low Fe spike emergence stage that results quite shares similarly metabolite compounds expressed during the spike development of High Fe and Low Fe genotypes. In cluster 4 found different metabolite pool between High Fe and Low Fe stage. In cluster 4 further divided into 7, 8 and 9. In cluster 7 showed similar metabolite pool in High Fe seed maturation and milking stage and found close to each other. In cluster 8 observed Low Fe pre pollination stage different metabolite pool from Low Fe pollination and Low Fe milking stage. In cluster 9 found similarly metabolite pool in Low Fe pollination and Low Fe milking stage. In dendrogram results said that High Fe five spike development stage shares close metabolite pool to each other and in Low Fe four spike development stage also shares similarly metabolite pool to each other except spike emergence stage of Low Fe genotype.

Similarly, total of 60 compounds of known structure, comprising sugars, sugar alcohols, fatty acids, amino acids, organic acids, phenols and sterols were identified in stem extracts of groundnut using GC-MS by Mahatma *et al.* (2017) [15]. They revealed that sugars and fatty acids were predominant in stem extracts as compared to other metabolites, heat map and dendrogram analysis to visualize the relative levels and relationships of metabolites. Sharma *et al.* (2017) [16] studied the effects of soaking time, germination time and temperature on the responses; total phenolics, total flavonoids and antioxidant activity for the biochemical enhancement of bioactive components of Kodo millet. They similar found the GC–MS analysis of raw and optimized Kodo millet samples revealed the presence of number of compounds Hexadecanoic acid, 9,Octadecenoic acid and Stigmasterol. The first dynamic metabolome of the developing grain of the elite Chinese bread wheat cultivar Zhongmai 175 was analyzed, using non-targeted gas chromatography/mass spectrometry (GC/MS) for metabolite profiling reported by Zhen *et al.* (2016) [17]. They said that clustering analysis and heat map representation of levels of 74 metabolites from grain development and found all 74 metabolites were evaluated and five expression patterns during dynamic metabolome changes in grain development.

PCA was used to summarize the multivariate metabolite data, capturing the variables that explained the greatest variation [principal components (PC)] in each treatment group. The key-contributing metabolites, as determined by their contribution to the PCA plots, can be identified by their pairwise score plots and loadings values, as summarized in the Figure 5 and 6. PCA showed that the expression patterns of the five developmental stages differed significantly. PC1 accounted for 40.9%, PC2 for 21.2%, PC3 for 11.8%, PC4 for 10.5% and PC5 for 9.2% of the variance, together amounting to 93.6% (Figure 5). In PCA score/loading plots found that the class of fatty acid, organic acid, sugar and sterol molecules including Octadecanoic acid, 9,12-Octadecadienoic acid, Tetradecanoic acid, alpha-D-Glucopyranoside, D-Glucose, Galactose, D-Fructose, 1,2,3-Propanetricarboxylic acid, Malic acid, Butanedioic acid and beta-Sitosterol (Figure 6). Zhen *et al.* (2016) [17] showed that the PCA expression patterns of the six wheat developmental stages differed significantly using gas chromatography/mass spectrometry

(GC/MS) for metabolite profiling. The developmental stages of grain could also be separated into distinct clusters according to the results of PLS-DA which further revealed distinct metabolic alterations between the different stages. Similarly, the quantitative data for the 48 metabolites were subjected to PCA to assess the overall experimental variation and to outline the differences in the metabolite profiles among varieties of proso millet identified by Kim *et al.* (2013) [9].

Table 1: Functional classification of number of compounds identified in GC-MS

Stage Name	Class						
	Amino acid	Fatty acid	Organic Acid	Sugar	Sugar Alcohol	Sterol, Polyamines and other	Total
HFeS1	2	3	11	14	1	9	40
LFeS1	3	5	9	9	1	7	34
HFeS2	2	5	9	12	2	8	38
LFeS2	2	4	13	10	2	10	41
HFeS3	3	12	10	10	2	9	46
LFeS3	3	10	14	10	3	10	50
HFeS4	2	9	11	9	2	15	48
LFeS4	3	9	14	17	1	10	54
HFeS5	3	9	13	6	4	12	47
LFeS5	3	11	11	10	4	8	47

Table 2: Metabolites showed in Heat map

Stage name	Metabolites of High-Fe Genotype	Metabolites of Low-Fe Genotype
Spike emergence	12	12
Pre pollination	8	8
Pollination	21	13
Milking	23	13
Seed maturation	17	10

Fig 1: Sample collection

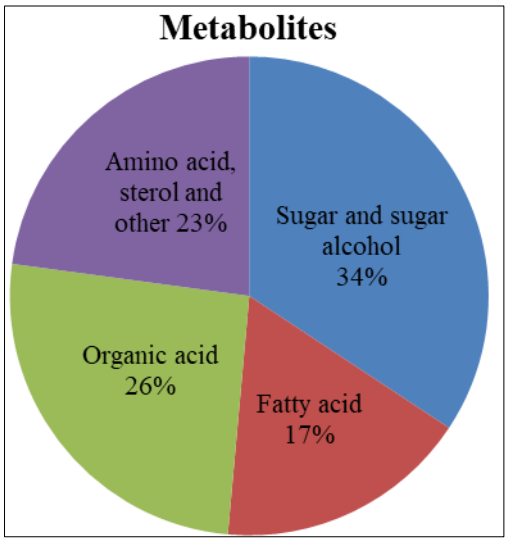

Fig 2: Pie charts shoes the classification of metabolites

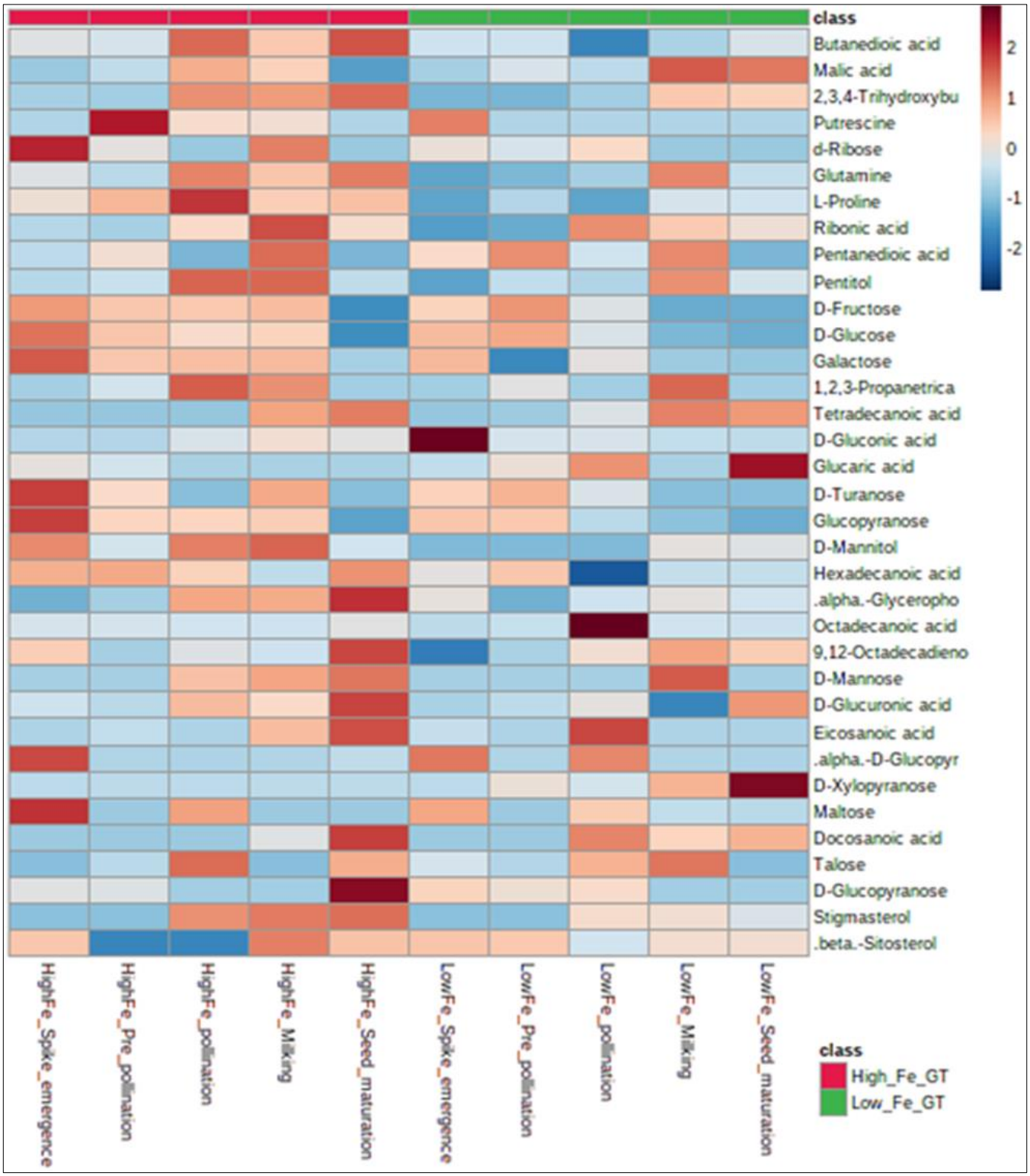

Fig 3: Heat map shows the common metabolite identified in spike development stages

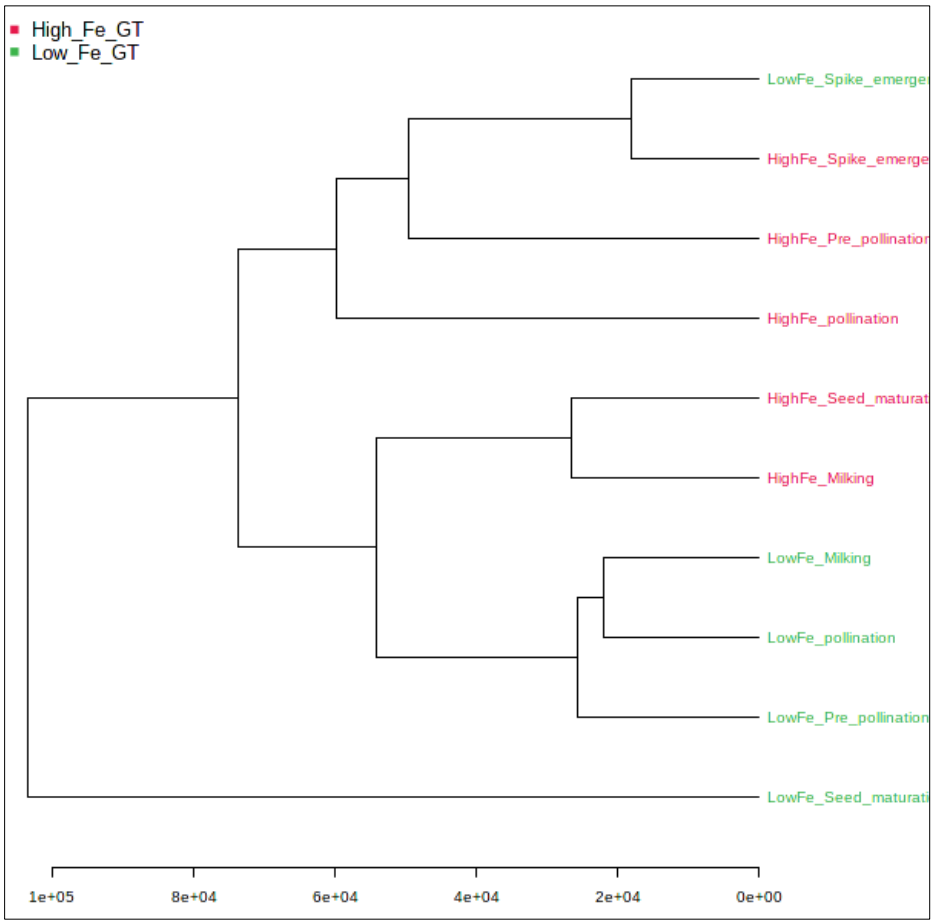

Fig 4: Clustering result shown as dendrogram

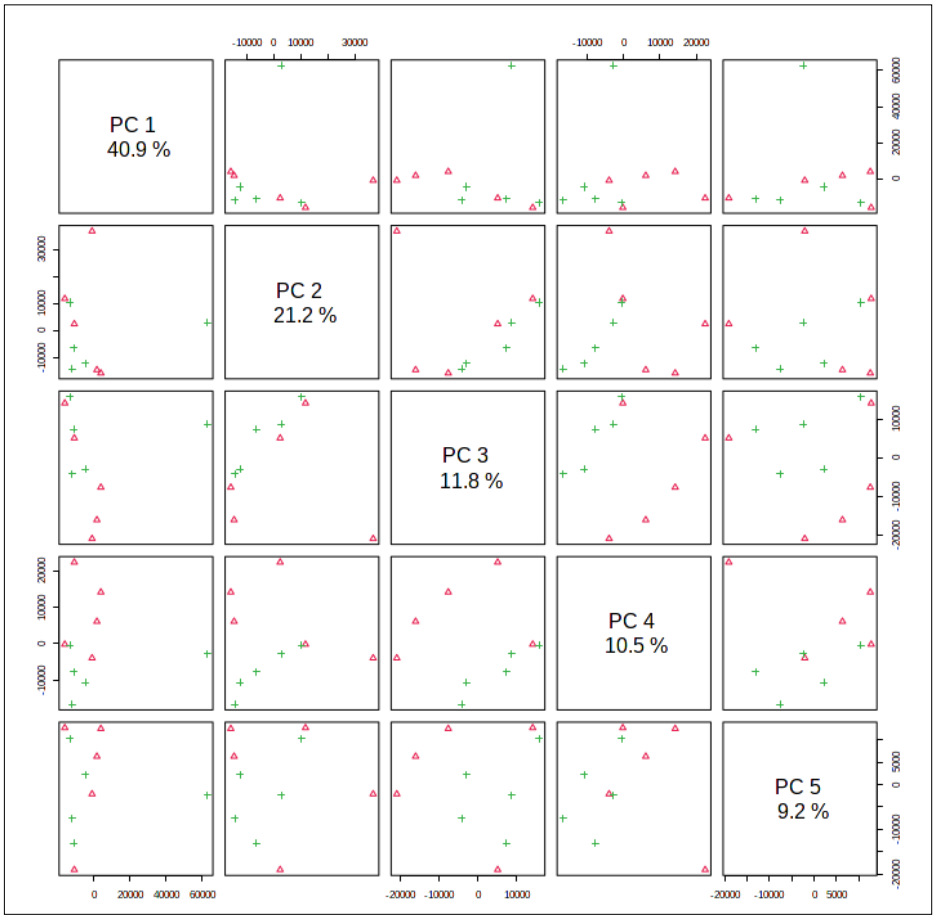

Fig 5: Score plots between the selected PCs. The explained variance of each PC is shown in the corresponding diagonal cell

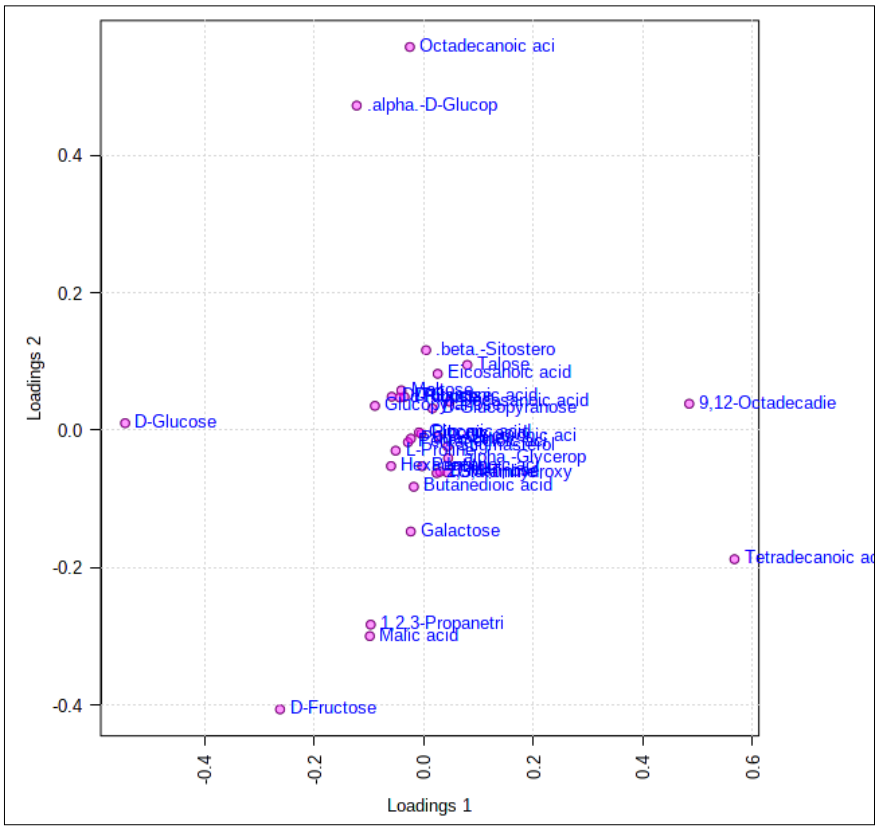

Fig 6: Shows the loading plot between the selected PC

Conclusion

Metabolome profiling identified dynamic changes in metabolite levels and correlations among such levels in spike development stages of iron content barnyard millet genotypes for developing seeds. Our comprehensive metabolic heat map and dendrogram results may be useful when breeding programs seek to improve grain quality.

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