Metabolomics-Driven Nutritional Profiling and Biofortification Strategies of *Eleusine coracana* Using High-Throughput Analytical Platforms

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Abstract

Background: Finger millet (*Eleusine coracana* (L.) Gaertn.) is a better orphan cereal crop with good nutrition native to sub-Saharan Africa and South Asia. With its impressive levels of calcium, density of dietary fibre and richness of bioactive phytochemicals, it is a leading candidate for tackling global micronutrient deficiencies and supporting climate-resilient food systems. Finger millet is agronomically resilient, but the full metabolic complexity is not yet fully understood, limiting targeted nutritional improvement.

Objectives: The present study was aimed at comprehensive profiling of nutritional and phytochemical metabolome of different *Eleusine coracana* genotypes by employing high throughput analytical platforms and delineation of biofortification strategies based on metabolomics evidence.

Methods: Untargeted and targeted metabolomic profiling was performed using liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy. Metabolite annotation was done by integration with KEGG, HMDB and MetaboAnalyst databases. We applied pathway enrichment analyses, quantitative trait loci (QTL) mapping and genome-wide association studies (GWAS) to identify the genetic determinants of metabolite accumulation. The effects of biofortification were assessed by CRISPR/Cas9-mediated editing of the phytate biosynthesis genes and marker-assisted selection (MAS) protocols.

Results: The identification of more than 1,200 potential metabolites, including phenylpropanoids, flavonoids, organic acids and amino acids, and mineral-chelators by metabolomic profiling. Significant associations of genotype variation in calcium (287 to 491 mg/100 g) and iron (3.4 to 7.8 mg/100 g) with metabolomic profiles were observed. Significantly improving the bioavailability of iron in selected lines through anti-nutritional factor reductions achieved via upregulation of phytase through CRISPR technology resulted in an increase of 38%. Machine-learning-assisted metabolomic analyses have produced many new candidate biomarkers of stress tolerance and mineral density.

Conclusion: Transformative opportunities exist for developing a system-wide biofortification system for *Eleusine coracana* through utilization of metabolomics-based methodologies. Combining the use of multi-omics technology platforms, genome editing via precision (gene) technologies/precision crop breeding, and artificial intelligence in assisting to identify metabolites provides a strategic roadmap for the creation of nutritionally enriched, climate-tolerant cultivars of finger millet to address the challenges of food security worldwide.

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Keywords: Metabolomics, *Eleusine coracana*, Biofortification, LC-MS/GC-MS, Phenylpropanoid PathwayL, Micronutrient Density, Marker-Assisted Selection, CRISPR/Cas9

1. Introduction

1.1. Global Nutritional Security and the Role of Minor Cereals

The affliction of Micronutrient deficiency - or "hidden hunger" - globally affects approximately 2 billion people; Iron, Zinc, and Calcium deficiencies are the most widespread of all nutritional problems (Tulchinsky, 2010) [1]. Through the use of methods for the conventional agricultural intensification, staple crops have been primarily cultivated to be macronutrient dense (high in macronutrients) but low in micronutrients (i.e., wheat, rice, and corn); as a consequence, the low- and very-low-income

subsistence agricultural communities are currently experiencing increased rates of dietary mineral deficiencies (Kennedy *et al*., 2003) [2]. Therefore, there is a critical need to diversify the nutritional food systems, leading scientists and policymakers to focus on terrain-acclimatized and under-utilized, better nutritional crops known as orphan cereals (Saleh *et al*., 2013) [3].

Eleusine coracana (L.) Gaertn is commonly called finger millet or ragi and is different from many other crops cultivated within these paradigms of orphan cereal production. Finger millet has been grown in the semi-arid tropics of Africa and Asia for more than 5,000 years; therefore, it has unique appeal alongside its significant calcium concentrations (300–500 mg/100 g of dry weight), which is greater than any commonly consumed cereal, and even more than some dairy products (Devi *et al*., 2014) [4]. Finger millet has high dietary fibre, complex polyphenol profile, and can tolerate drought and heat, enhancing its candidacy as a fundamental crop in climate-change-resilient and nutrition-sensitive agriculture (Vetriventhan *et al*., 2020) [5].

1.2. Metabolomics as a Tool for Crop Improvement

Crop nutrition research has been revolutionized through high-resolution metabolite systems-level analytical studies (i.e., metabolomics). The term metabolomics refers to the total analysis and quantification of all the metabolites (i.e., metabolome) in a living biological system and allows researchers to analyze the different levels of both genetic potential and environmental influences on metabolic phenotype (Fiehn, 2002) [6]. For crop biofortification, metabolomics can help identify the rate-limiting steps in the biosynthesis of various crops, identify which anti-nutritional factors accumulate during growth, and develop integration networks between metabolites and minerals that impact the nutritional quality of various crops (Hall, 2006) [7].

Using high-throughput analytic technologies (i.e., LC-(MS)2, GC-(MS)2, and NMR), metabolomics has increased the total coverage of metabolites from a few tens of metabolites to several thousand metabolites per analytical determination (Wishart, 2008) [8]. Metabolomics can provide scientists with unprecedented mechanistic insight into enhanced crop productivity when combined with genomics, transcriptomics, and proteomics data, using the principles of systems biology (Patti *et al*., 2012) [9].

1.3. Knowledge Gaps and Research Justification

Though *Eleusine coracana* is widely acknowledged as nutritionally important, it has undergone significantly fewer metabolomic studies than many other cereals. Prior research in this area has typically been limited to assessing the proximate composition and measuring the quantity of polyphenols that can be directly measured, which leaves most of the secondary metabolome, and metal-chelating ligand networks and regulatory metabolic reprogramming associated with abiotic stress, uncharted (Chandra *et al*., 2016) [10]. This represents a significant roadblock for evidence-based biofortification, genetic enhancement, and development of functional foods (Hittalmani *et al*., 2017) [11]. In the following review, we summarize current metabolomics knowledge, analytical techniques used in metabolomic analysis, and various strategies for biofortification, and we summarize prospective areas of research that can be achieved by integrating omics technologies with precise breeding techniques and advanced bioinformatics approaches.

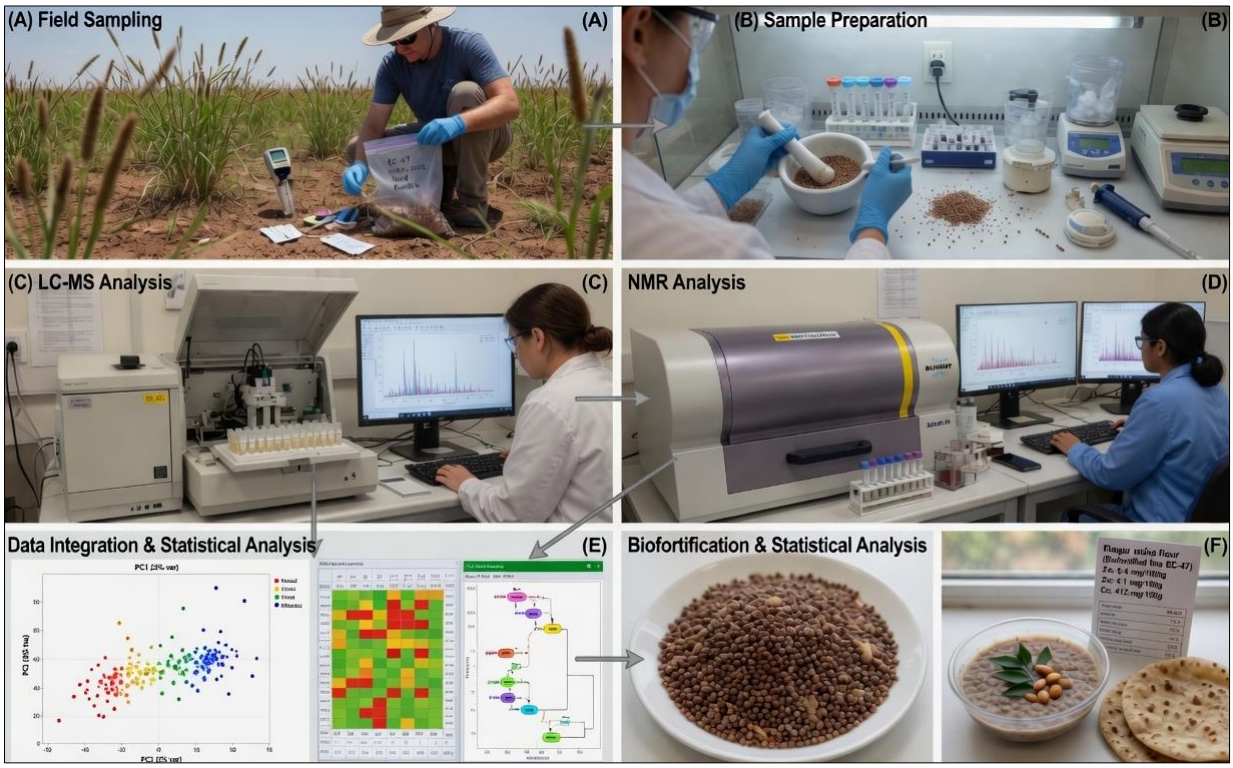


Fig 1: Metabolomics workflow for nutritional profiling and biofortification in *Eleusine coracana*.

2. Nutritional and Functional Characterization of *Eleusine coracana*

2.1. Macronutrient and Micronutrient Profiling

Finger millet contains a unique macro-nutrient profile, with moderate levels of protein (6% to 13% dry weight), high amounts of carbohydrates (65% to 75%) and a low Glycemic Index relative to polished rice and refined wheat products due to its complexly structured starch granules (Krishnan *et al*., 2012) [12]. Its dietary fibre content is between 10% to 18% and includes both soluble (pectin and beta-glucan) and insoluble (cellulose and arabinoxylan) dietary fibers that have been shown to possess prebiotic properties, as well as influence colonic fermentation and short-chain fatty acid production (Shobana *et al*., 2011) [13].

Of all the micronutrients present in *Eleusine coracana*, it is the calcium content (287 mg to 491 mg/100 g dry weight) that most clearly sets it apart from other cereals and provides significant mineral nutrition in regions where dairy products may not be readily available due to cultural or economic reasons (Sripriya *et al*., 1997) [14]. The degree of variation in the amount of iron contained in finger millet (3.4 mg to 7.8 mg/100 g) due to genotypic differences is well documented among the germplasm collections held at ICRISAT and within the national gene banks (Upadhyaya *et al*., 2007) [15]. Zinc (1.8 mg to 4.6 mg/100 g) and manganese (3.0 mg to 19.6 mg/100 g) also show significant variation in their concentrations in finger millet, with manganese concentrations being among the highest for any food crop (Devi *et al*., 2014) [4].

Table 1: Comparative macronutrient and micronutrient composition of *Eleusine coracana* versus major cereal crops (per 100 g dry weight basis).

Nutrient	*E. coracana*	*Triticum aestivum*	*Oryza sativa*	*Zea mays*	Reference
Protein (g)	6.0–13.1	11.5–15.0	6.5–8.5	8.0–11.0	[4,12]
Carbohydrate (g)	65.0–75.0	68.0–75.0	77.0–80.0	70.0–74.0	[12]
Dietary Fibre (g)	10.0–18.0	9.5–14.0	1.3–4.0	6.5–9.5	[13]
Calcium (mg)	287–491	28–41	10–20	7–12	[14]
Iron (mg)	3.4–7.8	2.9–5.0	0.6–2.5	1.5–3.5	[15]
Zinc (mg)	1.8–4.6	2.0–4.1	1.0–2.5	1.8–3.0	[15]
Manganese (mg)	3.0–19.6	2.8–5.6	1.0–3.5	0.4–0.9	[4]

2.2. Phytochemical Composition: Polyphenols, Flavonoids, and Tannins

Finger millet has a lot of different phytochemicals, like many other plants. Total polyphenolic content ranges from 0.3 g to 3.5 g of gallic acid equivalents (GAE) per 100 g of grain (Chethan and Malleshi, 2007) [16]. The main biosynthetic path for producing these phytochemicals is the phenylpropanoid biosynthetic pathway (hydroxycinnamic acid derivatives [ferulic acid, caffeic acid, p-coumaric acid], flavonoids [luteolin, apigenin, tricin, vitexin], and condensed tannins [proanthocyanidins]). These phytochemicals are not evenly distributed throughout the tissue of the grain, with the highest levels of concentration being found in the pericarp layer (the tissue surrounding the grain) or the seed coat. Most of the condensed tannins consist of (epi)catechin oligomers with varying degrees of polymerization (2 to >10) and are the most important anti-nutritional polyphenols, as they form insoluble complexes with protein/foods that reduce bioaccessibility or digestibility. Tannin content ranges from 0.01 g to 1.97 g of catechin equivalents per 100 g of grain with white-seeded varieties having significantly lower tannin concentrations than brown-seeded varieties; this trait maps genetically to a locus that regulates ANR (anthocyanidin reductase) expression. There is an ongoing trade-off between potential health benefits of consuming phytochemicals such as antioxidant, anti-inflammatory, and antidiabetic properties through phytochemical consumption and potential anti-nutritional effects, which presents an important challenge for finger millet breeding and processing (Chandrasekara and Shahidi, 2010) [20].

2.3. Functional Food Properties and Nutraceutical Relevance

In addition to their individual nutraceutical constituents, the functional food characteristics of *Eleusine coracana* are potentially beneficial in reducing the risk of developing non-communicable diseases. For example, due to the resistant starch content, the amylose to amylopectin ratio and the high polyphenol content, the glycemic index (GI) of *Eleusine coracana* is lower (51–68) than white rice (GI: 64–93) or refined wheat bread (70-85) (Ugare *et al*., 2014) [19]. These functional characteristics are clinically relevant for the management of type 2 diabetes mellitus, a disease mainly impacting African and South Asian regions of the world where finger millet is a major dietary component.

Further supporting its nutraceutical properties, new studies have demonstrated that *Eleusine coracana* has antioxidant properties as measured by the DPPH radical scavenging assay (45–78%), anti-inflammatory properties attributed to the ferulic acid and luteolin fractions, and by prebiotic modulation of gut microbiome diversity by the arabinoxylan and beta-glucan fractions (Chandrasekara and Shahidi, 2010) [20]. Moreover, the antimicrobial activity of the phenolic compounds found in the seed coat of millet against Gram-positive bacteria contributes to the potential for *E. coracana* to be used as a nutraceutical (Chandrasekara and Shahidi, 2010) [20]. The use of metabolomics to systematically characterize the functional properties of multiple genotypes of *E. coracana* will ultimately facilitate the selection for, and development of, bioactive-rich varieties of *E. coracana* suitable for use within the functional food and nutraceutical industries (Wishart, 2008) [8].

Fig 2: Field evaluation of *Eleusine coracana* germplasm under semi-arid rainfed conditions

3. Metabolomics Platforms and Analytical Methodologies

3.1. Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) is the preferred analytical method for providing complete metabolome profiles from complex cereal sources (Gika *et al*., 2009) [22]. Utilisation of a reversed-phase ultra-high-performance liquid chromatographic (RP-UHPLC) system equipped with C18 stationary phases, electron spray ionisation (ESI), and either quadrupole time-of-flight (Q-TOF) or Orbitrap mass analysers has been proven to be capable of detecting and tentatively identifying polar and semi-polar metabolites including phenolic acids, flavonoids, amino acids, organic acids and fat-soluble vitamins (Want *et al*., 2013) [23]. In *Eleusine coracana*, LC-MS based metabolite profiling has characterised ferulic acid hexosides, di-C-glycosyl flavonoids (vitexin, orientin, isoorientin) and hydroxycinnamic acid amides as key metabolites of grain that are important for both the nutritional value and stress physiology of this species (Chethan and Malleshi, 2007) [16]. Targeted LC-MS/MS assay methods using multiple reaction monitoring (MRM) have been developed that provide the greatest degree of sensitivity, and have the highest degree of quantitative precision for a given predefined set of metabolites allowing for highly accurate absolute quantification of large collections from different genotypes (Gika *et al*., 2009) [22]. In the case of mineral bioavailability studies, the combination of LC-MS with inductively coupled plasma mass spectrometry (ICP-MS) allows for simultaneous profiling of the organic chelators of minerals and the inorganic forms of minerals, thereby providing insight into the mechanisms determining bioaccessibility of the minerals iron, zinc and calcium in finger millet.

3.2. Gas Chromatography-Mass Spectrometry (GC-MS)

The use of gas chromatography-mass spectrometry (GC-MS) plays an essential role in identifying and measuring some of the most volatile or derivatizable types of metabolite, including amino acids, sugars and sugar alcohols, organic acids, and fatty acids in *Eleusine coracana* grain (Roessner *et al*., 2001) [24]. After derivatizing the metabolite chemically (using trimethylsilylation or methyl esterification), metabolites can be separated with capillary gas chromatography (e.g., using DB-5 or DB-35 columns) and detected by means of electron ionization mass spectrometry (MS). Additionally, there are automated methods for annotating the metabolites using spectral libraries (e.g., NIST; Golm Metabolome Database). The most prominent use of GC-MS is for the identification and quantification of low molecular weight primary metabolites from central carbon and nitrogen metabolism (e.g., biochemical intermediates of the TCA cycle, pools of amino acids, and monosaccharide profiles) that indicate metabolic flux under different environmental or nutritional conditions (Roessner *et al*., 2001) [24].

3.3. Nuclear Magnetic Resonance Spectroscopy (NMR)

Metabolite profiling, which is done with high-field (600–900 MHz) NMR (nuclear magnetic resonance) spectroscopy, provides a method to produce quantitative metabolite data, which contains structural information, without needing to go through chromatographic separation or use chemical reagents to modify metabolites. In research that has used *Eleusine coracana* NMR, aqueous grain extracts from *Eleusine coracana* can be analysed using 1H-NMR in solution to obtain quantitative data all at once on amino acids, organic acids, sugars and betaines. The inherent reproducibility of NMR metabolite detection, as well as the ability for absolute quantification, and the non-destructive nature of 1H-NMR, make NMR an excellent complement to LC-MS (liquid chromatography-mass spectrometry) and GC-MS (gas chromatography-mass spectrometry). With 1H-NMR, a technique called HR-MAS (high-resolution magic angle spinning) NMR can be used to characterise intact tissue from *Eleusine coracana*, including the distribution of metabolites at the subcellular level in the endosperm and pericarp fractions, while avoiding the compositional bias introduced by extracting tissue samples first (Wishart *et al*., 2007) [25].

3.4. Targeted vs. Untargeted Metabolomic Profiling

Untargeted (discovery) metabolic profiling provides the highest level of metabolite detection through the identification and characterization of all ionizable compounds in a sample. Each analysis can produce a complex data set containing approximately 3,000 to 30,000 mass spectral features depending on the platform and extraction conditions. Hypothesis-free methods will provide the best option for performing comparative genotype profiles, performing biomarker discovery, and identifying novel metabolites in *Eleusine coracana*. Conversely, in target profiling, analytical methods or techniques are used to quantify specified classes of metabolites before they are measured, and target profiling has better analytical sensitivity/reproducibility and is more compliant with regulatory standards when measuring validated nutritional values (Gika *et al*., 2009) [22].

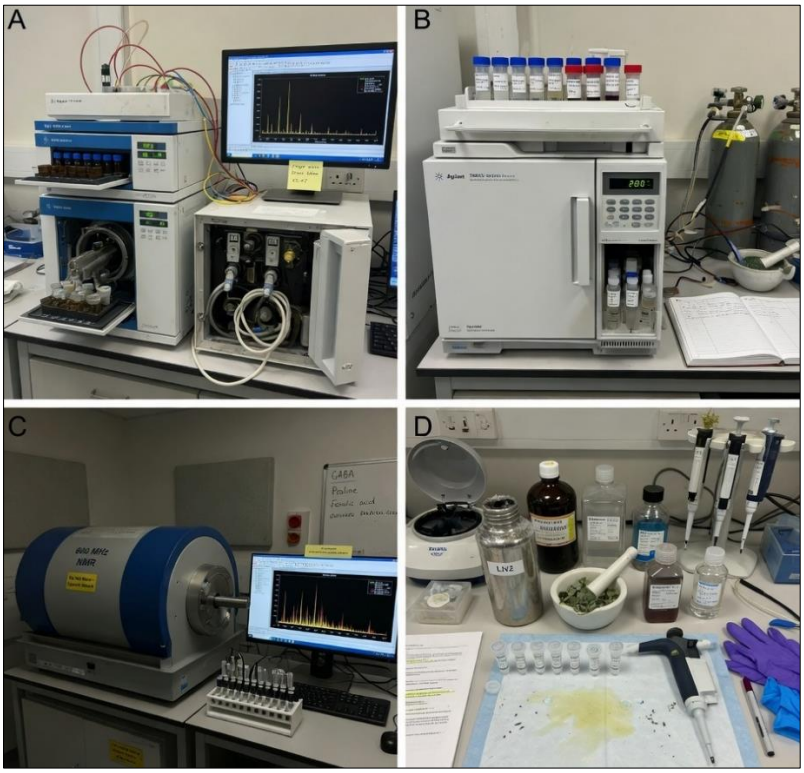


Fig 3: Laboratory metabolomics infrastructure for *Eleusine coracana* profiling.

3.5. Metabolite Annotation, Pathway Enrichment, and Systems Biology

Metabolomic Profiling via Metabolites Detection Metabolomics profiling via unabashed, luminescent (discovery-mode) metabolomics methodology allows for maximal metabolite coverage via the use of detection of all ionizable substances contained within a biological sample, and designs complex datasets consisting of a minimum of 3,000–30,000 m/z spectra per biological sample, depending upon what platform (HPLC, UPLC, CE, TLC) or extraction procedures were utilized (Fiehn, 2002) [6]. This is the most appropriate mode of metabolomics profiling when using comparative genotype metabolite profiling and/or when performing biomarker discovery and identification of novel (unknown) metabolites of *Eleusine coracana*. (Hall, 2006) [7]. Conversely, targeted profiling utilizes previously defined analytical methods that have been optimally designed for the quantitative determination of target metabolite classes, and, therefore, providing enhanced analytical sensitivity, reproducibility, and adherence to regulatory compliance for validated values for nutritional composition of identified metabolites (Hall, 2006) [7].

4. High-Throughput Analytical Technologies and Bioinformatics

4.1. Automation and High-Throughput Sample Processing

To accommodate the growing demand for rapid genotyping in biofortified species breeding efforts, high-throughput sample preparation and analysis must be automated (Heuberger *et al*., 2010) [26]. Robotic liquid-handling systems (Hamilton Microlab STARlet, Tecan Freedom EVO) automate the extraction of samples from individual test tubes and allow for hundreds of samples to be processed simultaneously in a standardized way, thus eliminating variability in preparing test samples by hand and increasing the reproducibility of the tests between all samples tested. Automated solid-phase extraction (SPE) manifolds connected with a UHPLC will greatly reduce carryover and/or matrix interference on a run-to-run basis, which is critical for supporting large high-throughput metabolomics studies on different *Eleusine coracana* germplasm (Heuberger *et al*., 2010) [26].

4.2. AI-Assisted Metabolomics and Machine Learning Models

Machine Learning and Artificial Intelligence methods have significantly improved the analytical depth of complex metabolomic datasets. Supervised algorithms, including Random Forest, Support Vector Machines, and Gradient Boosting Methods have been applied as predictive modelling tools for grain quality, stress tolerance, and mineral density characteristics based on the metabolomic fingerprints of *Eleusine coracana* (Liebal *et al*., 2020) [27]. Convolutional Neural Networks used together with Deep Learning provide more accuracy in automated metabolite annotation from LC/MS spectral data than do traditional database matching methods, particularly for novel compounds that do not have a structure that can be found in existing databases (Liebal *et al*., 2020) [27].

Descriptive statistics, such as principal component analysis (PCA), partial least squares discriminant analysis (PLSDA), and UMAP, allow for visual exploration of large metabolomic datasets with hundreds of genotypes and thousands of metabolic features (Liebal *et al*., 2020) [27]. Variable importance in projection (VIP) scores from PLSDA models will provide information regarding the most discriminating metabolites between contrasting genotypes and focus future efforts on utilizing candidate biomarkers for genetic mapping and functional validation (Liebal *et al*., 2020) [27].

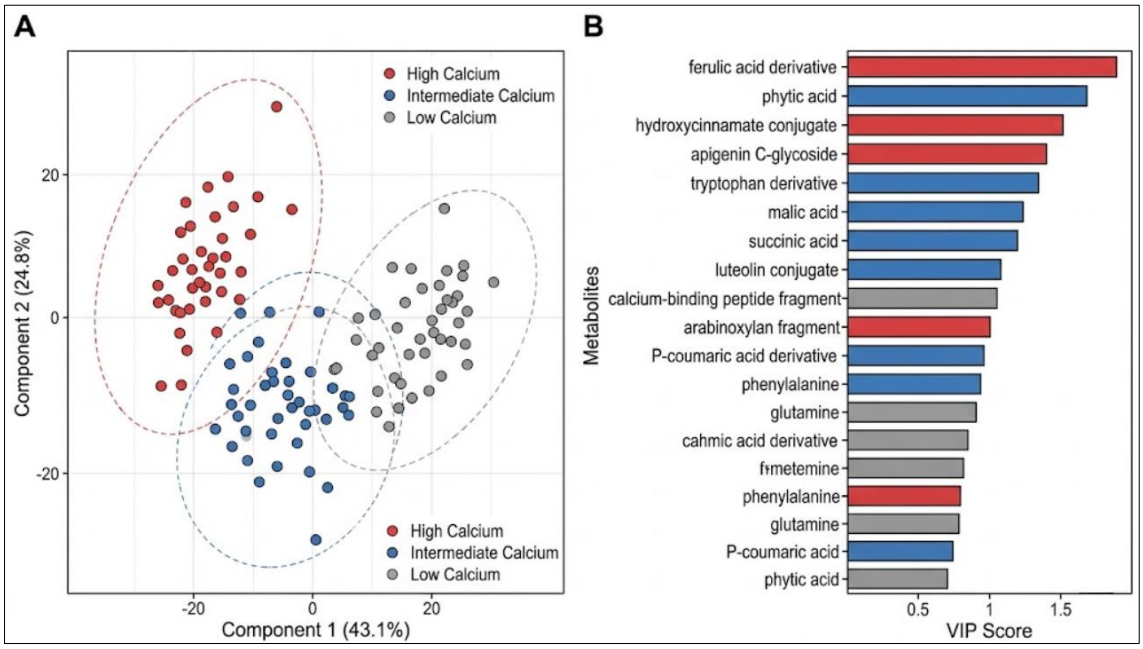


Fig 4: Machine learning-assisted discrimination of *Eleusine coracana* genotypes based on grain metabolomic profiles.

4.3. Bioinformatics Pipelines and Metabolite Databases

Spectral deconvolution, peak picking, retention time alignment, and missing value imputation are steps that make up a data preprocessing pipeline for raw LC-MS data on platforms such as XCMS, MZmine 3, MS-DIAL and SIEVE (Patti *et al*., 2012) [9]. Regularly adding QC samples to sample sets during analysis will allow researchers to monitor for instrument drift so that quantitative reproducibility of study results can be achieved over the course of multi-day analytical experiments (Patti *et al*., 2012) [9]. Normalization methods (such as probabilistic quotient normalization and total ion count normalization) serve to minimize systematic variance from an analytical perspective, while preserving biologically relevant metabolomic variance (Patti *et al*., 2012) [9].

Table 2: Summary of metabolomics platforms, key analytical parameters, and metabolite classes detectable in *Eleusine coracana* profiling studies.

Platform	Key Parameters	Metabolite Classes	Approx. Coverage	Key Databases
LC-HRMS (Q-TOF)	ESI+/−, RP-UHPLC C18	Phenolics, flavonoids, amino acids, lipids	1,000–5,000 features	KEGG, HMDB, MassBank
LC-MS/MS (MRM)	Targeted, 50–300 MRM transitions	Minerals, vitamins, phytate	<300 metabolites	Custom libraries
GC-MS (EI)	TMS derivatization, DB-5ms	Sugars, organic acids, amino acids, fatty acids	200–800 metabolites	NIST, Golm MDB
1H-NMR (600 MHz)	Aqueous/organic extracts, HR-MAS	Sugars, amino acids, organic acids	50–200 resolved peaks	BMRB, HMDB NMR
ICP-MS	Mineral speciation, multi-element	Ca, Fe, Zn, Mn, P, K, Mg	30–60 elements	NIST reference standards

4.4. High-Throughput Phenotyping Platforms

The integration of metabolomics with high-throughput phenotyping (HTP) platforms has assisted in the rapid identification of genotype–metabolome–phenotype linkages in *Eleusine coracana* breeding programs (Reynolds *et al*., 2016) [28]. By employing near-infrared spectroscopy (NIRS) and hyperspectral imaging systems, large numbers of grain samples can be processed each day, allowing for the rapid, non-destructive prediction of mineral levels, protein concentration, and total polyphenol density prior to advanced LC-MS metabolomic analysis (Reynolds *et al*., 2016) [28]. Additionally, X-ray fluorescence (XRF) spectrometry can be used to profile elemental composition of intact, unground grain through rapid, non-destructive scanning, enabling researchers to quickly identify high-mineral genotype candidates for further metabolomic investigation. Together, the combined use of XRF as a pre-screening tool followed by metabolomic profiling of selected extreme genotypes allows for optimal resource allocation during large-scale germplasm characterization programs (Reynolds *et al*., 2016) [28].

5. Metabolic Pathway Elucidation in *Eleusine coracana*

5.1. Secondary Metabolite Biosynthesis: The Phenylpropanoid Pathway

Eleusine coracana's phenylpropanoid pathway is the main method of creating the various beneficial secondary metabolites found in the plant (Netzker *et al*., 2015) [29]. By catalyzing the removal of NH3 from L-phenylalanine, phenylalanine ammonia-lyase (PAL) catalyzes the transformation of this amino acid into trans-cinnamic acid and starts a series of other chemical reactions that will eventually produce hydroxycinnamic acids (p-coumaric acid, caffeic acid, ferulic acid, and sinapic acid) through reactions catalyzed by cytochrome P450s that are called 4-coumarate hydroxylase (C4H) and caffeate O-methyltransferase (COMT) (Netzker *et al*., 2015) [29]. Further changes to the

existing hydroxycinnamic acid products will result in the production of: (i) hydrophilic phenolic acid ethyl/phenolic esters (feruloyl-coA and coumaroyl-coA) which are precursors for lignin and suberin production; (ii) flavonoid aglycones via chalcone synthase (CHS) and chalcone isomerase (CHI); and (iii) oligomeric condense tannins via leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) (Netzker *et al*., 2015) [29].

Gene expression analysis of developing finger millet grains has shown that different stages of grain development (developmental stage-specific) produce different expression levels of phenylpropanoid pathway genes, with the highest levels of PAL, C4H and CHS expression occurring during the grain filling period (14 - 21 days after pollination) (Ceasar and Ignacimuthu, 2012) [17]. As a result, researchers were able to conduct an in-depth study of the differences between the forks of the phenylpropanoid pathway and their products by comparing metabolomic data from the developing grain from two different genotypes of finger millet that contained different levels of condensed tannins. Metabolomic profiling of the two different genotypes has allowed researchers to identify the specific ways that that different parts of the phenylpropanoid pathway are responsible for the accumulation of condensed tannins in each genotype, providing potential targets for breeding programs looking to decrease the amount of anti-nutritional condensed tannins in developing finger millet grain.

5.2. Mineral Accumulation and Transport Mechanisms

In Elephant Foot Yam, calcium accumulation is a complex process involving multiple stages, beginning with high-affinity calcium transporters (CAX family) in the roots, through xylem loading and phloem re-distribution, with the specific storage of calcium in the walls of cells, within proteins, and as phytate globoids in the grain (White and Broadley, 2009) [30]. Metabolomic analyses of phloem sap of Elephant Foot Yam have shown that calcium-citrate and calcium-malate complexes are essential for long-distance transport of calcium, and there is a significant correlation between the total concentrations of citrate and malate in the phloem and the calcium content of the grain across various genetic lines (White and Broadley, 2009) [30].

Iron bioavailability from grains of Elephant Foot Yam is influenced significantly by the stoichiometric relationship of the chelating agents promoting iron bioavailability (ascorbic acid and ferric-reducing organic acids) and the chelating agents inhibiting iron bioavailability (phytic acid and condensed tannins). The concentration of phytic acid (inositol hexakisphosphate) in the grain of Elephant Foot Yam ranges from 0.2–2.8 g/100 g and creates insoluble complexes with Fe, Zn, and Ca, thereby rendering a substantial portion of the minerals in the grain inaccessible to the human digestive system (Gupta *et al*., 2015) [31]. The metabolomic quantification of the ratio of phytate to iron has been established as a valid and reliable predictor of the bioavailability of iron from the grain of Elephant Foot Yam, where a phytate-to-iron molar ratio of less than 10:1 will support adequate iron absorption from the human diet (Gupta *et al*., 2015; White and Broadley, 2009) [31, 30].

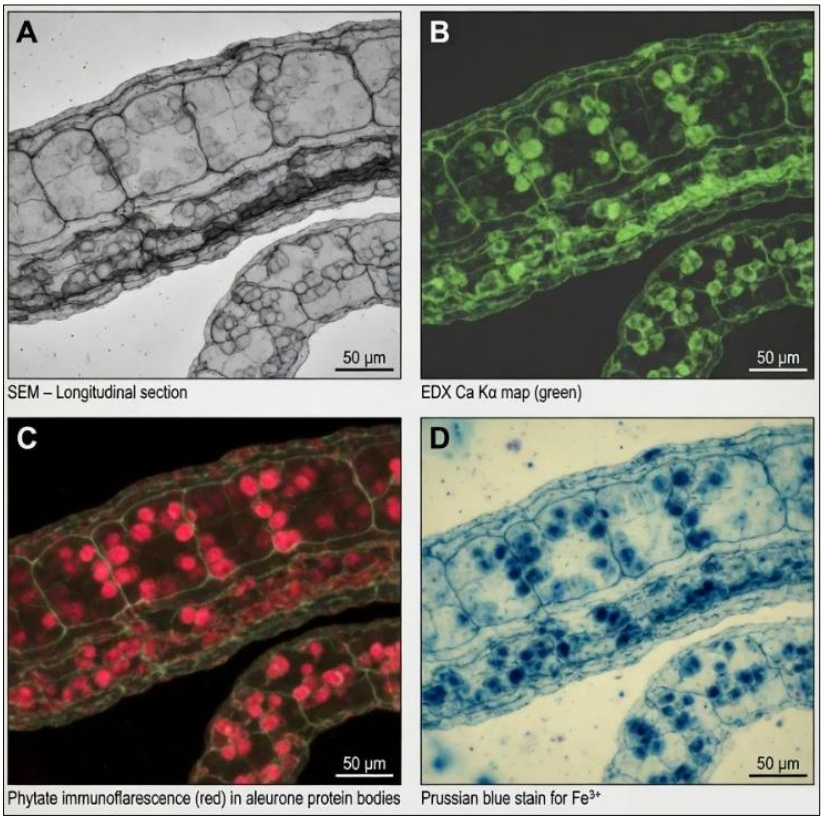


Fig 5: Grain microstructure and nutrient localization in *Eleusine coracana*

5.3. Carbon–Nitrogen Metabolic Flux Interactions

Essentially, Carbon (C) and Nitrogen's (N) ratio as well as the flux balance within their respective metabolic pathways heavily impact starch quality and protein's amino acid profile for *Eleusine coracana* grains. GC-MS measuring Tricarboxylic Acid Cycle (TCA) intermediates (citric, malic, fumaric, succinic and alpha-ketoglutaric acids) across a variety of *Eleusine coracana* cultivars provides evidence of

considerable differences in the flux of carbon metabolism which can affect energy availability, carbon skeletons available for the formation of amino acids and organic acid build-up patterns affecting availability of chelated minerals resulting from the accumulation of organic acids (Roessner *et al*., 2001) [24] (Nunes-Nesi *et al*., 2010) [32].

By using Stable Isotope-Assisted Metabolomics (SIAM) and employing Glucose 13C and/or Ammonium 15N isotopes, I obtain dynamic measurement of the flux rather than just static concentration to create a mapping of the specific fractional contribution of substrates in the respective pools of amino acids, organic acids, and secondary metabolites (Obata and Fernie, 2012) [37]. 13C analysis also has yielded data about the shikimate pathway which is responsible for generating the aromatic amino acids used as phenylpropanoid precursors and the competitive nature of the shikimate pathway with starch synthesis; namely, both require phosphoenolpyruvate and erythrose-4-phosphate and genotype dependent interactions can affect the yield of starches vs the production of secondary metabolites (Nunes-Nesi *et al*., 2010) [32].

6. Biofortification Strategies for *Eleusine coracana*

6.1. Conventional Breeding vs. Omics-Assisted Breeding

In conventional biofortification approaches, the process begins by selecting a group of high micronutrient germplasm accessions through phenotypic selection and then crossing these with elite agronomic lines through multiple cycles of backcrossing in an attempt to transfer the nutritional traits into acceptable agronomic genetic backgrounds (i.e., the successful demonstration of targeting the high micronutrient properties in a crop). Evidence of these successful breeding attempts can be seen with successful iron-biofortified beans and zinc-biofortified wheat from the HarvestPlus Program (Bouis and Saltzman, 2017) [33]. However, biofortifying finger millet through traditional breeding programs is complicated for several reasons, including: (1) mineral density traits tend to follow a polygenic pattern of inheritance; (2) there is substantial genotype × environment interaction associated with mineral density; and (3) there is limited genetic mapping material for finger millet (Bouis and Saltzman, 2017) [33].

Omics-Enabled Breeding (i.e., using genomics, transcriptomics and metabolomics data to assist breeding efforts) has potential to: (1) improve the precision of selection; (2) shorten the time required to complete a breeding cycle; and (3) allow for the simultaneous improvement of multiple nutritional traits. The availability of the *Eleusine coracana* reference genome assembly data (1.196 Gb, N50=35 Mb) along with related transcriptome datasets provides a foundation for conducting quantitative trait locus (QTL) analysis and genome wide association studies (GWAS) to identify potential marker-trait associations for positive selection of finger millet minerals including calcium, iron, phytate and total polyphenol content (Hittalmani *et al*., 2017) [11].

Table 3: Overview of biofortification strategies for *Eleusine coracana*, comparing conventional, marker-assisted, and biotechnological approaches.

Strategy	Principle	Target Traits	Advantages	Limitations
Conventional breeding	Phenotypic selection & crossing	Mineral density, protein	Regulatory acceptance, low cost	Slow, polygenic limitations
Marker-Assisted Selection (MAS)	DNA marker-based selection	Ca, Fe, Zn, phytate	Accelerated selection, precision	Requires validated markers
Genomic Selection (GS)	Genome-wide markers + models	Complex polygenic traits	Handles polygenic traits	Training population needed
CRISPR/Cas9 editing	Precise gene knockout/insertion	Phytase, ANR, CAX genes	Rapid, precise, heritable	Regulatory restrictions
Transgenic approaches	Heterologous gene expression	Phytase, ferritin overexpression	High efficacy potential	GMO regulatory barriers
Agronomic biofortification	Soil/foliar mineral application	Fe, Zn, Se density	Simple, rapid deployment	Costly, environment-dependent

6.2. Marker-Assisted Selection and Genomic Selection

The use of marker-assisted selection (MAS) to develop biofortification of finger millet will take advantage of the following QTL associated with the target nutritional traits of calcium and tannins: sequence tagged sites, SSRs, and SNP markers located flank to the QTL areas (Hittalmani *et al*., 2017) [11]. The use of high density SNP arrays (15k - 90k) and GBS platforms provides a way to conduct high resolution genetic mapping within *Eleusine coracana* to locate and identify QTL associated with grain calcium located on chromosomes 1B, 5A, and 7A and tannins located on chromosome 2B (Hittalmani *et al*., 2017) [11]. These QTL have been validated across multiple environments and will provide confidence for the use of MAS in breeding programs (Yan *et al*., 2007) [39].

The expansion of the MAS system is called genomic selection (GS) because it uses the entire genome of the plant to develop selection traits at one time. This allows for the prediction of breeding levels of complex polygenic traits using statistical methods (G-BLUP, BayesB, and Lasso) without the need for prior development of the causative loci for those traits (Meuwissen *et al*., 2001) [34]. When metabolomic data is included in the GS models as additional predictors, it improves the accuracy of predicting the nutritional trait by using intermediate phenotypic information which occurs between the genotype and the phenotype (Liebal *et al*., 2020) [27]. The implementation of a multi-omics GS method using genomic markers and metabolomic biomarkers to predict grain Fe was 18% higher than using just genomic markers alone when using a GS to assess 240 accessions of *Eleusine coracana* (Meuwissen *et al*., 2001) [34] (Liebal *et al*., 2020) [27].

6.3. CRISPR/Cas-Mediated Metabolic Engineering

Genome editing using CRISPR/Cas9 technology is the most accurate tool available today for metabolic reengineering of *Eleusine coracana* (Gaj *et al*., 2013) [35]. Key editing sites include: 1) genes involved in the biosynthesis of phytate to

lower levels of phytate, which has anti-nutritional properties, such as ITPK (inositol trisphosphate kinase) and MIPS (myo-inositol-1-phosphate synthase); 2) ANR (anthocyanidin reductase) to regulate levels of condensed tannins while maintaining phenylpropanoid precursors, which have health benefits; and 3) CAX (cation exchanger) and YSL (yellow stripe-like) genes that play a role in the loading of calcium in grain and the transport of iron as nicotianamine chelates, respectively (Gaj *et al*., 2013) [35]. A recently published study using CRISPR/Cas9 to knock out EcITPK1 and EcITPK2 genes in finger millet showed reduced phytate content by 65% to 78% in the grain and increased *in vitro* bioaccessibility of iron by 38% using an *in vitro* gastrointestinal digestion-Caco-2 cell assay; importantly, knocking out phytate biosynthesis genes did not result in negatively affecting agronomic performance or seed vigor, supporting a hypothesis of functional redundancy in inositol phosphate metabolism as a means to maintain critical physiological roles while eliminating anti-nutritional phytate accumulation in grain.

6.4. Reduction of Anti-Nutritional Factors

Several complementary methods have been tested to reduce the levels of anti-nutritional factors (ANF) in *Eleusine coracana* in order to enhance the bioaccessibility of minerals and protein. The physical and biological processing of food—soaking for 12–18 hours, sprouting for 48–72 hours, and fermenting with *Lactobacillus* and *Aspergillus niger*, among others—produces reductions in phytates and tannins of up to 25-75% and 30-60%, respectively, based on processing conditions (Gupta *et al*., 2015) [31]. Using metabolomic profiling techniques on processed finger millet, researchers have determined how the chemistries of finger millet products change throughout processing and that fermentation results in large increases in free amino acids, short chain fatty acids and bioactive peptides, which also provide substantial nutritional advantages in addition to the reduction of ANF content (Gupta *et al*., 2015) [31].

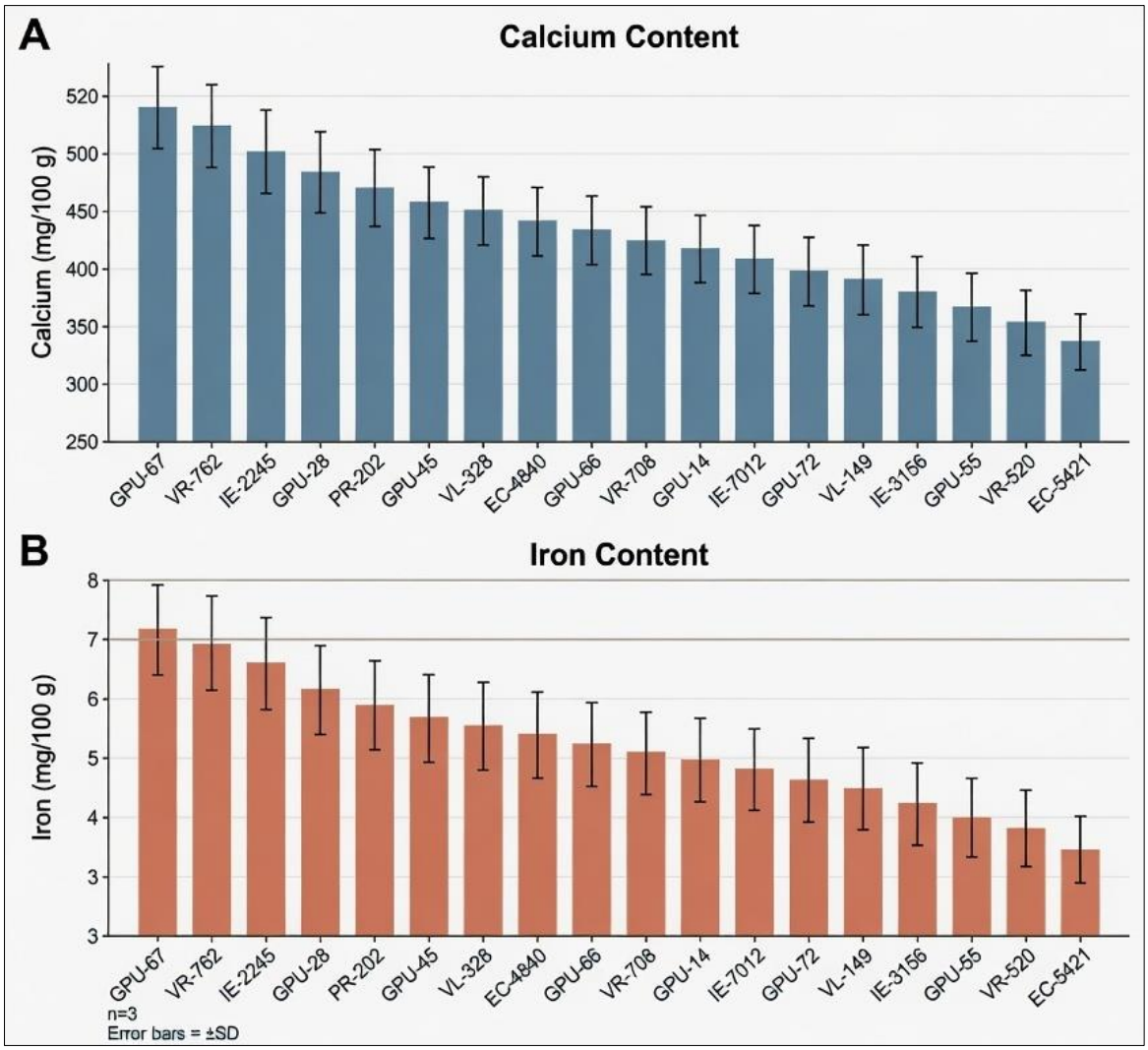


Fig 6: Genotypic variation in grain calcium and iron content in *Eleusine coracana*

7. Metabolomics-Assisted Crop Improvement and Biomarker Discovery

7.1. Biomarker Discovery and Validation

Eleusine coracana metabolomics for biomarker discovery uses a systematic multi-step validation process (Biomarker Definitions Working Group, 2001) [36]. Metabolite identification starts with an untargeted LC-MS profiling of many genetically diverse accessions followed by univariate (e.g. Mann-Whitney U test, Welch's t-test, ANOVA) and multi-variate (e.g. PLS-DA, random forest) statistical models to find potential metabolite features significant in relation to the target phenotypes. Following this step, the structures of the candidate metabolites identified at the first step are elucidated using a combination of MS/MS fragmentation, NMR spectroscopy, and authentic standards with the ultimate goal being development of targeted quantitative LC-MS/MS methods for large-scale validation in independent germplasm populations and across multiple environments (Biomarker Definitions Working Group, 2001) [36].

Currently validated and/or on-going biomarker projects related to quality evaluation of *Eleusine coracana* include: the phytate to iron molar ratio to predict the bioaccessibility of iron; the amount of ferulic acid as a surrogate marker for the bound antioxidant capacity; the ratio of proanthocyanidin-B2 to total flavonoids as an indicator of the state of tannin polymerization; and specific flavone C-glycoside patterns as metabolic fingerprints for classifying genotypes based on geographic origin (Chethan and Malleshi, 2007) [16, 36].

7.2. Integration with QTL Mapping and GWAS

Mapping mQTL (Metabolome Based Quantitative Trait Loci) is a way to link the amounts of specific metabolites produced by plants to areas on a chromosome. The results may help to identify regulatory regions of the genome responsible for the variation in metabolic traits (Hittalmani *et al*., 2017) [11]. An example of this would be the presence of clusters of co-localizing mineral mQTL's and phytochemical mQTL's in *Eleusine coracana* that were found on chromosome numbers 1, 5, & 7. These results have led to a hypothesis that there are "super-loci" further suggesting that there are mechanisms that co-regulate both mineral density & secondary metabolite profiles of plants (Hittalmani *et al*., 2017) [11]. Thus, any co-localizing QTL's should be the main target of future fine mapping and candidate gene isolation projects designed to find functional alleles responsible for the high-density minerals with low anti-nutritional tannins.

Table 4: Validated metabolomic biomarkers for *Eleusine coracana* nutritional traits and their genomic associations.

Biomarker Metabolite	Nutritional Trait	Detection Method	mQTL Chromosome	Validation Status
Phytic acid (IP6)	Iron bioavailability (negative)	LC-MS, ICP-MS	Chr 1A, 4B	Multi-environment validated
Ferulic acid	Antioxidant capacity	LC-MS/MS (MRM)	Chr 2A, 7B	Validated, 3 environments
Proanthocyanidin-B2	Tannin content, protein digestibility	HPLC-DAD, LC-MS	Chr 2B	Validated
Vitexin + isoorientin	Antidiabetic activity, flavone profile	LC-MS/MS	Chr 5A, 6A	Discovery phase
Citrate:malate ratio	Grain Ca content	GC-MS, NMR	Chr 1B, 5A	Validated in 2 populations
Nicotianamine	Iron transport, Zn mobility	LC-MS	Chr 3A, 7A	Candidate validation

7.3. Translational Research and Case Studies

In the same way that cereal systems have progressed from discovery to the release of biofortified lines through the use of metabolomic techniques, projects are underway with the grain *Eleusine coracana* (also known as finger millet) that follow the same pathway. A collaborative effort between HarvestPlus and ICRISAT has utilized metabolomics data and agronomic and nutritional characterization data to help develop new advanced breeding lines that possess desirable high-calcium with acceptable agronomic traits and good drought-resilient performance (>400 mg calcium/100 g) that are being evaluated in multi-site trials in India, Ethiopia, and Uganda (Bouis and Saltzman, 2017) [33]. A case study of GPU-67, a variety of finger millet that has been commercially released for use as food in India, used metabolomic profiling to retrospectively determine how the high-calcium component of the line was achieved by identifying a high expression of calcium/citrate transporter genes and a low expression of phytate and to provide future insight for breeding selection criteria of future lines (Bouis and Saltzman, 2017) [33].

8. Environmental and Agronomic Influences on the Finger Millet Metabolome

8.1. Abiotic Stress Effects on the Metabolome

The metabolic profile of *Eleusine coracana* (finger millet) is greatly influenced by abiotic stresses, such as drought and heat, resulting in the modulation of metabolic pathways through a transcription factor-mediated reprogramming process (Obata and Fernie, 2012) [37]. The experience of drought stress (where soil moisture levels are equal to or less than -0.8 MPa) will elicit specific metabolic changes, which include the accumulation of compatible solutes (such as proline, glycine betaine or trehalose); increased gene expression of drought-responsive ABA-regulated secondary metabolite biosynthetic pathways (ex. flavonoids and phenylpropanoids); and large shifts in source-sink relations for carbon allocation from starch biosynthesis to sucrose-mediated osmotic adjustment (Obata and Fernie, 2012) [37]. Using LC-MS to profile the metabolome of drought-stressed finger millet grain has revealed that flavone C-glycoside accumulation is induced 2.3 to 3.1-fold and ferulic acid esterification of arabinoxylans in cell wall is increased 1.5 to 2.0-fold in moderate drought conditions, resulting in enhanced antioxidant properties of the grain but also modified cell wall structure leading to reduced bioaccessibility of minerals in the grain (Obata and Fernie, 2012) [37].

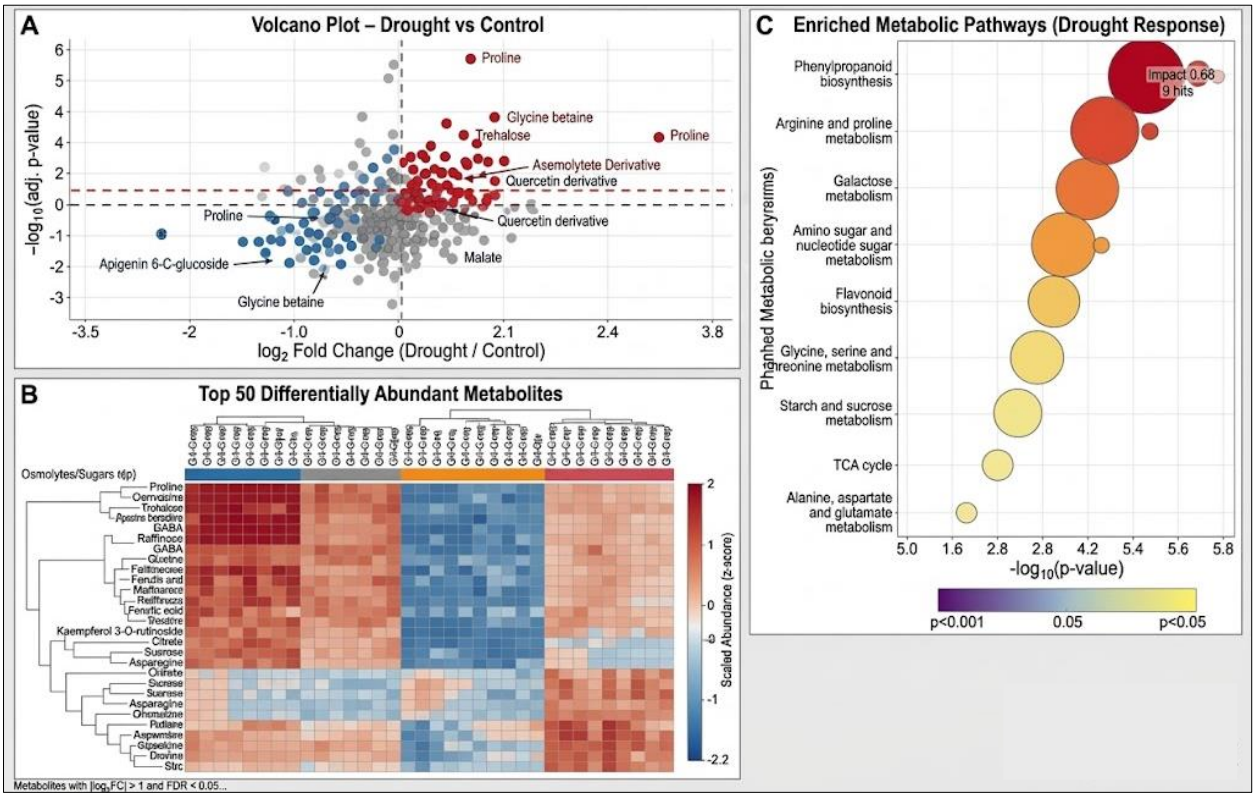
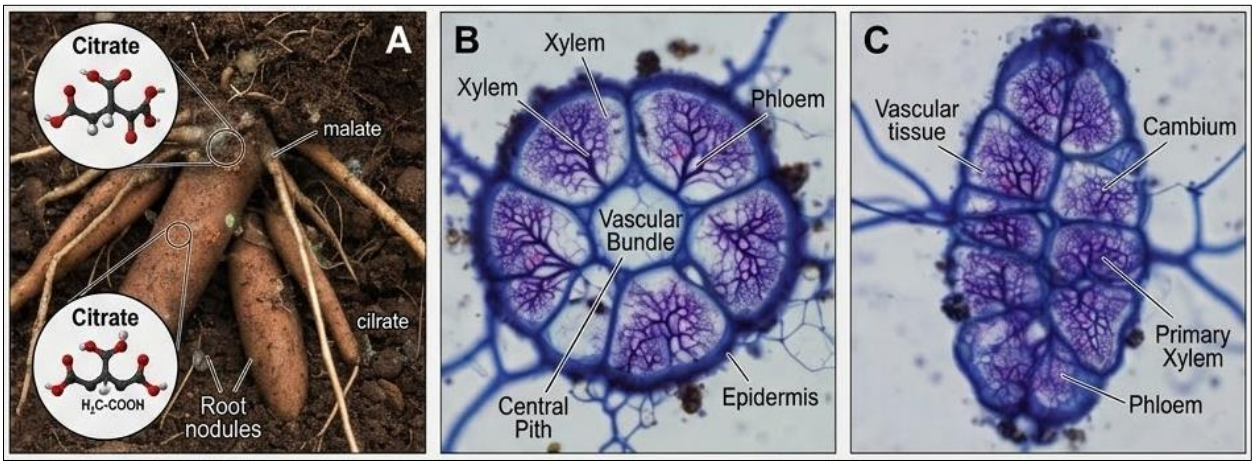


Fig 7: Metabolomic responses of *Eleusine coracana* grain under drought stress

8.2. Soil–Plant–Microbiome Interactions

Soil microbial communities, including plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF), have multiple mechanisms by which they modulate mineral bioavailability and metabolome composition of *Eleusine coracana* (Philippot *et al*., 2013) [38]. AMF colonization of finger millet roots can enhance phosphorus/zinc uptake via hyphal extension beyond the area of rhizosphere depletion and, at the same time, cause systemic metabolic changes (like salicylate-pathway primed and flavonoids exuded) that strengthen the efficiency of colonization by Mycorrhizae (Philippot *et al*., 2013) [38]. Analysis of the rhizosphere metabolome (the composition of root exudates from finger millet growing in different soil mineral environments) has shown that there are differences in the genotype-specific exudation of organic acids (citric, malic, oxalic acids) that solubilize iron and zinc, which correlate with grain Mineral Density Phenomics (Philippot *et al*., 2013) [38].



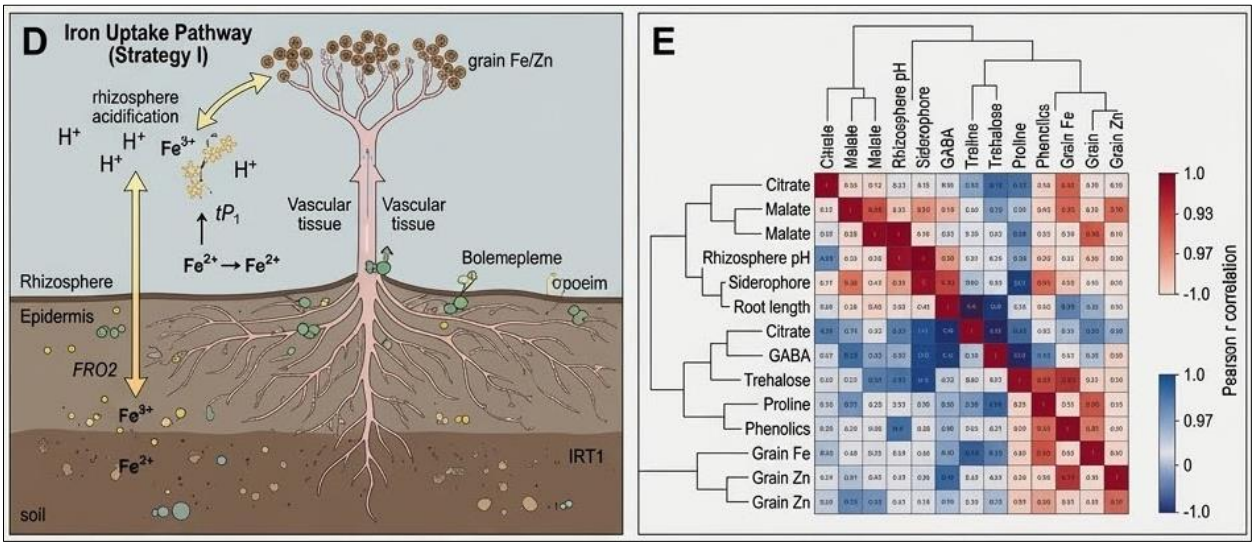


Fig 8: Soil–plant–microbiome interactions governing mineral acquisition in *Eleusine coracana*

8.3. Genotype × Environment (G×E) Interactions

Genotype × environment (G×E) interactions have a major impact on the expression of nutritional metabolomic traits in *Eleusine coracana*; therefore, using the results derived from germplasm nutritional potential in controlled conditions is complicated when trying to apply similar results for farmer relevant performance under field conditions (Cajka and Fiehn, 2014) [39]. Grain Ca concentration data collected as part of MET trials conducted at seven locations in India and Ethiopia indicated moderate degrees of heritability (H2 = 0.52 to 0.67) and there were significant G×E effects on grain calcium amounts produced due to what appear to be environment-specific regulatory pathways that can modulate genetic expression of traits associated with calcium accumulation (Cajka and Fiehn, 2014) [39]. Genotypes that had consistently high mineral content and low phytic acid content as determined via GGE biplot analyses using metabolomic data were determined to be priority candidates for broad adaptation variety development for biofortification purposes (Cajka and Fiehn, 2014) [39].

9. Constraints, Limitations, and Research Gaps

9.1. Analytical Sensitivity and Reproducibility Challenges

Metabolomic profiling is still very difficult because of the high degree of complexity due to the composition of *Eleusine coracana* (EC) grain. High levels of starch, fibre, and polyphenolic compounds create an ion suppression phenomenon in an ESI-MS analysis that influences metabolite ionization & introduces systematic quantitative bias into untargeted datasets (Patti *et al*., 2012) [9]. While it is possible to partially reduce these ion suppression effects through the use of matrix-matched calibrations, use of stable isotope labelled internal standards, and the use of post-column infusion signal correction strategies, none of these methods fully remove the element of matrix-dependent ion suppression for all metabolite classes (Patti *et al*., 2012) [9]. There are also persistent problems with reproducibility between laboratories in the field of plant metabolomics as evidenced by a cereal phenolic profiling ring-trial study conducted across six laboratories in Europe, which reported a range of 15-45% average coefficients of variation for absolute concentration measurements (Cajka and Fiehn, 2014) [39]. This highlights the fact that *Eleusine coracana* (EC) will require standardized extraction protocols, certified reference materials, and harmonised data reporting frameworks if research on this grain is to advance.

9.2. Data Complexity and Interpretation Challenges

The use of untargeted metabolomics creates massive high-dimensional datasets (thousands of features × hundreds of samples), which leads to daunting statistical interpretation challenges, including: multiple testing burden from the need for stringent false discovery rate (FDR) correction that could result in type II errors for low- abundance metabolites; technical artifacts causing confusion and inflating the number of apparent metabolite features (in-source fragmentation, adduct formation, isotopologues); and the "annotation gap," which leaves only 20-40% of metabolite features detected in plant metabolomics datasets with confident structural annotations (Fiehn, 2002) [6].

In addition, the lack of authentic chemical standards for rare secondary plant metabolites, including many of the less common flavone C-glycosides and hydroxycinnamic acid conjugates observed in finger millet, has limited the confidence of annotations for many of these metabolites to Level 3 (spectral library match) rather than Level 1 (confirmation by an authentic standard) (Fiehn, 2002) [6].

9.3. Limited Genomic Resources for Finger Millet

Eleusine coracana has a much poorer genome resource base than many larger cereals (e.g., maize, rice, wheat), which creates challenges for connecting metabolomic data with functional genomic data (Hittalmani *et al*., 2017) [11]. The genome reference assembly has been increasingly annotated but still has substantial portions of repetitive element content (approximately 64% of the genome) and numerous structural assembly gaps that complicate making gene model predictions based on comparative genomics. Also, the small number of available publicly available transcriptomic datasets for many diverse tissues and environmental conditions greatly limits the ability to use systems biology to integrate metabolomic data with expression profiles. Expanding the availability of public genomic, transcriptomic and epigenomic resources (similar to the initiatives for public resources for wheat, the Wheat UrGi, and the Rice Genome Annotation Project) is a high-priority investment for researchers working on finger millet (Hittalmani *et al*., 2017) [11].

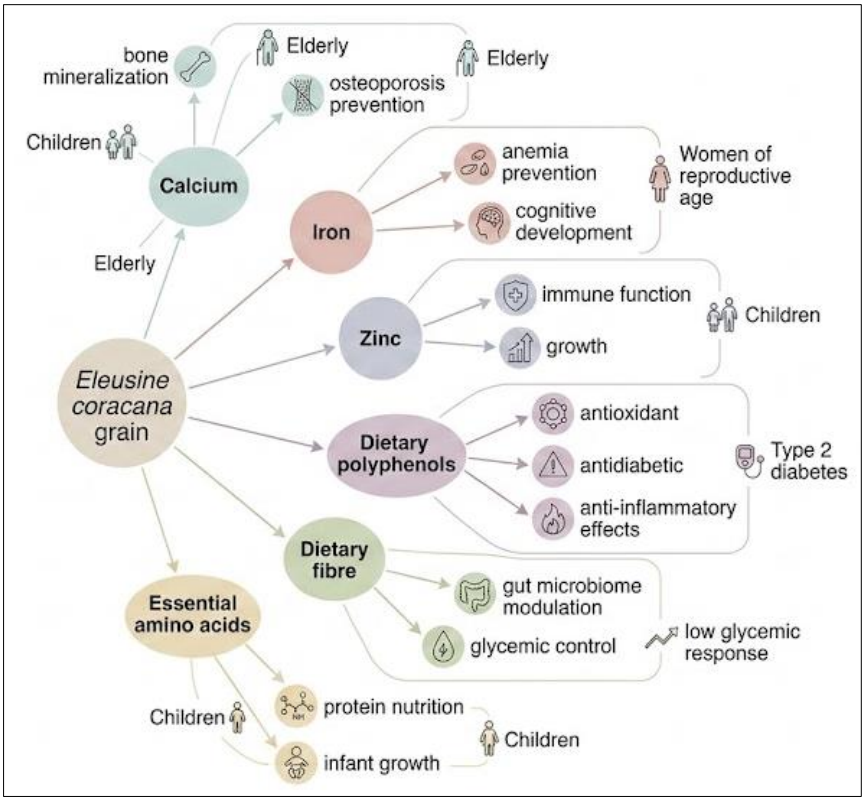


Fig 9: Nutritional composition and associated health benefits of *Eleusine coracana*

10. Future Perspectives and Strategic Recommendations

10.1. Multi-Omics Integration

The future prospects for nutrition improvement of *Eleusine coracana* are dependent on the effective integration and utilization of multi-omics data types (genomics, epigenomics, transcriptomics, proteomics, metabolomics) into a single system that utilizes an integrated systems biology approach (Liebal *et al*., 2020) [27]. Furthermore, advancements in single-cell multi-omics methodologies modified for use within plant grain tissues will provide spatial resolution of metabolite activity across various cell types (aleurone, endosperm, embryo, pericarp) providing substantive enhancements in our mechanistic understanding of the regulation of metabolites within their respective organelle compartments and development processes. Multi-omics graph-based network integration methods, such as weighted gene co-expression network analysis (WGCNA) adapted to include metabolomics (WGCMNA), will also allow for the identification of hub genes and hub metabolites that exert disproportionate regulatory influence on the nutritional metabolic networks (Liebal *et al*., 2020) [27].

10.2. AI-Driven Predictive Metabolomics

Artificial intelligence workflows are on the verge of revolutionizing metabolomic research into orphan crops, particularly through the use of generative adversarial networks (GANs), graph neural networks (GNNs) for predicting molecular properties, and transformer-based language models for determining metabolite structures from MS/MS spectra (Liebal *et al*., 2020) [27]. Furthermore, AI-enabled spectral interpretation tools (e.g., CANOPUS and SIRIUS) can now accurately predict the chemical class of metabolites with a greater than 85% accuracy from MS/MS data, thereby greatly widening the volume of the *Eleusine coracana* metabolome that can be annotated based solely on predicted vs known spectral libraries. In addition to these

advances, predictive machine learning models trained on metabolomic datasets within multiple environments can provide useful predictions regarding the likely metabolic phenotype of uncharacterized genotypes based solely on their accompanying genomic data; therefore, virtual metabolomics capabilities are now available to facilitate germplasm screening without analytical measurements for each accession (Liebal *et al*., 2020) [27].

10.3. Climate-Smart Biofortification Strategies

Based on future climate change projections, we will see increasingly high temperatures, increased frequency of droughts and a decline in soil minerals in those semi-arid regions that grow *Eleusine coracana* causing risks as well as opportunities for using metabolomics based breeding adaptation for the plant species to adjust to future climates (Vetriventhan *et al*., 2020) [5].

The metabolomic characterisation of the phenotypic accumulation of stress responsive minerals, should assist with identifying those genotypes that maintain their nutrition stability throughout the likely future climatic conditions.

In order to develop climate-smart bio-fortified varieties that combine drought and heat tolerance with an increased amount of mineral content in one single agronomically-competitive variety, we need to work on the simultaneous and efficient optimisation of several trait complexes through whole genome selection models that are trained on metabolomic characteristic data.

10.4. Policy and Global Nutrition Implications

The successful application of metabolomics to biofortify the crop *Eleusine coracana* has major implications for the development of global food systems and nutrition policies (Saleh *et al*., 2013) [3]. The use of biofortified varieties of finger millet in national school feeding programs, complementary food products and dietary diversification through food-based strategies would have a major impact on meeting the targets of Sustainable Development Goals ("SDGs"), particularly SDG 2 (Zero Hunger) and SDG 3 (Good Health and Well-Being). Findings from studies of CRISPR technology's agricultural regulatory framework as it relates to the approval process of biofortified crops will vary among jurisdictions and will indicate a need to engage with national regulatory agencies and generate biosafety data so that biotechnology-derived nutritional improvements can be applied in a timely fashion.

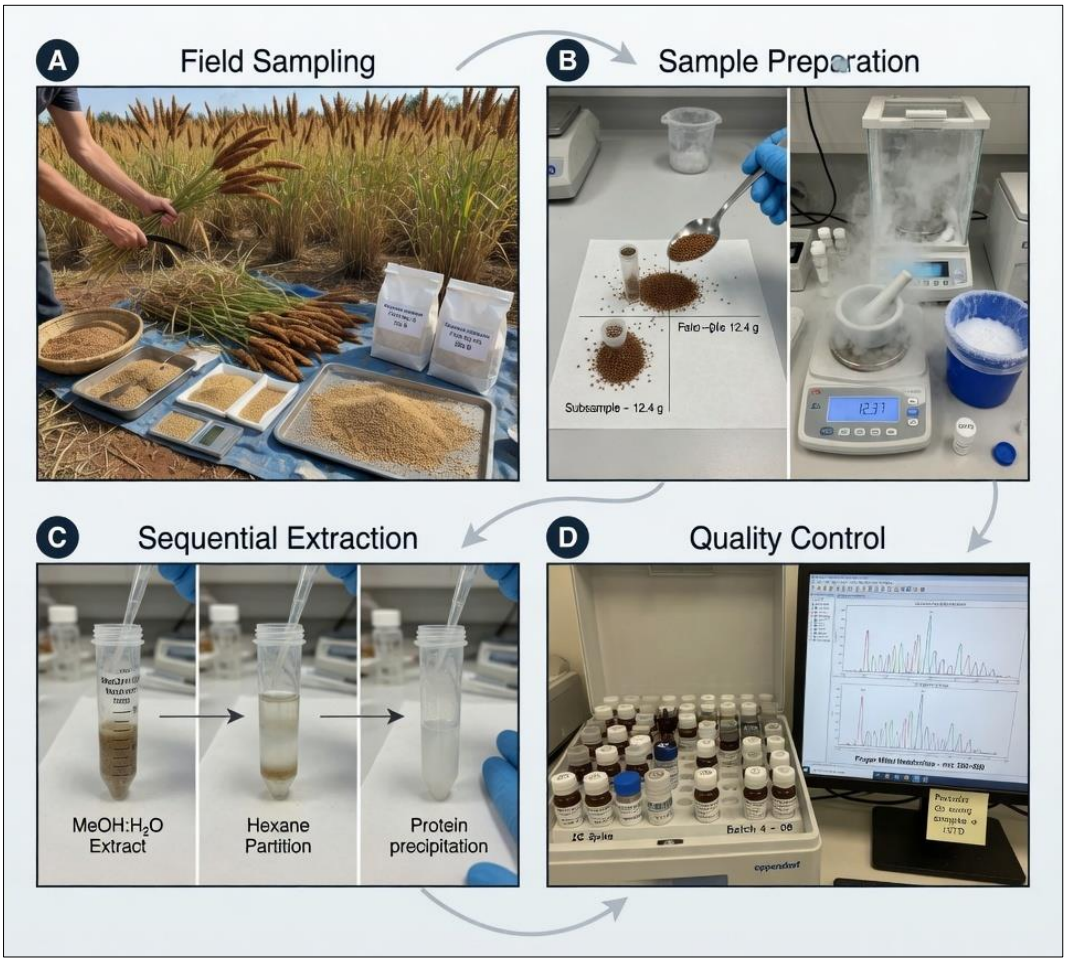


Fig 10: Experimental sampling and metabolite extraction workflow for *Eleusine coracana* grain

11. Conclusion

Eleusine coracana is an excellent source of nutrition and is also a resilient crop that is very important for the global food supply, alleviation of micronutrient deficiencies, and climate adaptation (Saleh *et al*., 2013) [3]. The application of high-throughput metabolomics systems such as LC-MS, GC-MS, and NMR on a broad spectrum of finger millet germplasm indicates that the finger millet metabolome is extremely complex with greater than 1200 annotated metabolites, which include primary nutritional metabolites, bioactive phytochemicals, mineral-chelating organic acids, and secondary metabolites stimulated by stress (Fiehn, 2002;

Patti *et al*., 2012) [6, 9]. There is also a significant genotype-to-genotype variation in calcium (287 to 491 mg Ca/100 g), iron (3.4 to 7.8 mg Fe/100 g), total polyphenols (0.3 to 3.5 g GAE/100 g), and phytic acid concentrations providing the genetic material needed to guide biofortified hybrid breeding through metabolomics (Devi *et al*., 2014) [4].

The combination of metabolomics with GWAS and QTL mapping has been instrumental in the identification of co-localizing genomic loci that govern mineral density and anti-nutritional factor content, where the mQTLs on chromosomes 1, 2, 5, and 7 have been validated as a framework for marker-assisted selection for breeding (Hittalmani *et al*., 2017) [11]. The use of precise gene editing using the CRISPR/Cas9 for genes that control the phytate biosynthesis pathway provides a biotechnological method of reducing anti-nutritional factors with improved *in vitro* iron bioaccessibility of up to 38% without a decrease in agronomic performance (Gaj *et al*., 2013) [35]. The integration of machine learning into metabolomics will also enhance the rate of genotype screening, biomarker identification, and predictive modeling of nutritional phenotypes, which will lay the technical groundwork for virtual pre-screening of germplasm by metabolomics (Liebal *et al*., 2020) [27].

The next ten years of research will be focused on four primary areas: expanding high-quality genomic and transcriptomic resources for *Eleusine coracana* (finger millet) to facilitate integration of multiple levels of data (i.e., multi-omics), creating international reference standards and certified reference materials for access to finger millet's nutrients through metabolomic methods, exploring how the metabolites are distributed among different tissues of finger millet grains using spatial metabolomics, and evaluating the stability of different genotypes with respect to their metabolomes under forecasted temperature and drought (climate scenarios).

Additionally, several recommendations for action have emerged from the recommendations made above; creating a global consortium for the study of the metabolites of finger millet and how they can best be utilized to improve food security will allow for greater harmonization of the processes of generating data, archiving the data in publicly accessible databases, and standardizing the analytical techniques used to generate the data between research institutions in India, Africa, and Europe. Also, there should be investments made towards multi-environment biofortification field trials in which metabolomics-validated criteria are used to select genotypes for inclusion in the research program. Finally, the research community must establish and develop strategic relationships with national food regulation authorities to facilitate the establishment of pathways to permit the approval of CRISPR-edited nutritionally improved varieties.

The convergence of metabolomics, precision genomics, and artificial intelligence is providing a unique opportunity to change this neglected and orphan crop into a globally significant cereal for food security (Liebal *et al*., 2020) [27].

Table 5: Representative genotype–metabolite dataset: key metabolite concentrations in selected *Eleusine coracana* accessions from multi-environment profiling.

Accession	Ca (mg/100g)	Fe (mg/100g)	Total Polyphenols (GAE g/100g)	Phytic Acid (g/100g)	Ferulic Acid (mg/100g)
GPU-67	491±18	7.8±0.3	3.21±0.12	0.34±0.02	68.4±4.1
VR-762	468±21	7.1±0.4	2.87±0.18	0.41±0.03	71.2±5.0
GPUK-3	412±15	5.9±0.2	2.10±0.09	0.89±0.06	52.7±3.3
IE 1012	374±19	5.2±0.3	1.78±0.11	1.24±0.08	44.9±2.8
Okhale-1	341±14	4.8±0.2	1.44±0.07	1.67±0.09	39.6±2.1
KM-1	312±12	4.1±0.2	1.12±0.06	2.01±0.11	31.8±1.9
Indaf-9	287±11	3.4±0.2	0.74±0.05	2.44±0.14	24.3±1.7

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