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Assessing the Genetic Potential of Micronutrients Accumulation in Pigeonpea Grains [*Cajanus cajan* (L.) Millsp.]

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ABSTRACT

Biofortification is a promising strategy for enhancing the micronutrient content of staple crops to combat hidden hunger. Pigeonpea [*Cajanus cajan* (L.) Millsp.], a climate-resilient legume, has significant potential to address nutritional deficiencies by providing essential micronutrients such as calcium (Ca), magnesium (Mg), iron (Fe) and zinc (Zn). This study evaluated 185 reference set accessions and 15 elite pigeonpea genotypes of diverse maturity groups from early (121–150 days) to late maturity (> 180 days) for their grain and cotyledon (dal) micronutrient content. Field trials were conducted during rainy 2023 at three diverse locations. Significant variation (Prob.Chisq < 0.0001) was observed among genotypes, with a two-fold difference in grain calcium content (GCaC) (1678.22–2506.27 ppm) and grain magnesium content (GMgC) (1327.04–2106.89 ppm), while grain iron content (GFeC) (30.46–34.22 ppm) and grain zinc content (GZnC) (27.83–36.10 ppm) exhibited moderate variation. GCaC and GMgC showed high estimates of heritability ($h^2 = 76.00\%$). Following the cluster analysis, 200 genotypes were grouped into five unique clusters. The largest cluster, Cluster II, had 72 genotypes, followed by Clusters III and IV, both of which had 43 genotypes. Principal component analysis revealed the key micronutrients contributing to genetic variation among the studied pigeonpea genotypes. The accumulation pattern of micronutrients in whole grain followed the order: Ca > Mg > Zn > Fe, whereas a dominant consumable form (dal) showed the trend of Fe > Zn > Mg > Ca. Direct selection for high cotyledon micronutrient would be an effective strategy over selection based on whole grain micronutrient content. The identified micronutrient-specific and dense genotypes can be used as potential donors for establishing biofortification breeding in pigeonpea.

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1 | Introduction

Food and nutritional security are the major global concerns. Despite advancements in agriculture and food production, inadequate access to nutritious food and malnutrition still remain unresolved. A healthy diet provides the right balance of essential micronutrients, including calcium (1000 mg/day), magnesium (440 mg/day for men, 370 mg/day for women), iron (19 mg/day for men, 29 mg/day for women) and zinc (17 mg/day for men, 13 mg/day for women) (Sheoran et al. 2022). Yet, two billion people suffer from micronutrient deficiencies worldwide, leading to serious health issues (WHO 2021). Lack of these micronutrients wanes the body, resulting in anaemia, fragile bones, poor immunity and muscle weakness. A daily cereals–legume diet is regular across the population strata. Enriching the nutritional composition of the staple cereals and legumes forms a powerful solution to address the challenges. Known for its dense protein packing, could legumes supply a substantial amount of essential micronutrients was the hypothesis envisioned. Pigeonpea, being the daily diet of the majority of households in semi-arid tropics, was found apt for the study.

Being a dryland legume, it meets both food and nutritional demands. Native to the Indian subcontinent, this hardy legume thrives in rain-fed regions, providing a crucial source of protein for more than a billion people across Asia and Africa (Mukherjee et al. 2023). Being a major protein as well as micronutrients source, it significantly contributes to diverse global food systems (Wu et al. 2024). Additionally, it offers essential vitamins such as B (B1, B2, B3 and B6), C and E, making it a well-balanced dietary choice. Each 100 g serving of pigeonpea contains 62.78 g of carbohydrates and 1.49 g of fat (ICMR-NIN 2020).

A typical pigeonpea grain is composed of three key structural components: cotyledons, seed coat and an embryo. The embryo is particularly rich in albumin and globulin, while the cotyledons serve as a major source of carbohydrates, protein and micronutrients (Mukherjee et al. 2023). However, a minor percentage of protein and micronutrients is also found in the seed coat too. Predominantly consumed as split cotyledon (dal) and whole grain accounts for 85%–100% of nutritional supply at consumption. While 15% of remnants are captured in the seed coat, it forms a nutritive feed.

With this background on pigeonpea micronutritional composition, a study was undertaken to explore the genetic potential of four major micronutrients: Ca, Mg, Fe and Zn. Though the earlier reports (Susmitha et al. 2022) suggested the grain micronutrients in pigeonpea, an in-depth understanding was required. Henceforth, exclusive research was carried out to systematically characterize the genetic variability and diversity for micronutrients (Ca, Mg, Fe and Zn) in the Pigeonpea International Genome-Wide Association Panel (PI-GAP) (Varshney et al. 2017) in order to identify the trait donors. The validated donors will then be deployed in the breeding pipeline as well as utilized to understand the molecular basis of micronutrient accumulation in grain and its components. Further findings will be projected to initiate the biofortification breeding in pigeonpea, paving the way for global nutritional security.

2 | Materials and Methods

2.1 | Genetic Material

The current study used 200 pigeonpea accessions comprising 185 reference set accessions and 15 elite genotypes from the PI-GAP, sharing the origin across 25 countries. The seeds were sourced from the Rajendra Singh Paroda Genebank at ICRISAT, India. This panel was selected for its extensive genetic diversity across multiple traits, including maturity duration, seed coat colour, seed weight, morphological characteristics and yield-related traits. The panel covered a broad range of maturity, classified into four distinct groups, namely, early (121–150 days: 45 genotypes), mid-early (151–165 days: 37), medium (166–180 days: 50 genotypes) and late-maturing (> 180 days: 68 genotypes) (Table S1).

2.2 | Field Experiment

The experiment was conducted during the rainy 2023 at three distinct locations, namely, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)-Patancheru, Regional Agricultural Research Station (RARS)-Warangal and Indian Institute of Pulse Research (IIPR)-Kanpur. The environmental conditions at each site varied as follows: ICRISAT-Patancheru (17.51° N latitude, 78.27° E longitude, 545.00 m above mean sea level) recorded an average maximum temperature of 31.8°C and a minimum of 21.50°C. The average rainfall during the cropping season was 923.18 mm, with a relative humidity (RH) of 86.33%. RARS-Warangal (15.50° N latitude, 79.28° E longitude, 268.50 m above mean sea level) had an average maximum temperature of 32.50°C and a minimum of 21.55°C, with 952.08 mm of average rainfall and RH of 85.97%. While, IIPR-Kanpur (26.27° N latitude, 80.14° E longitude, 125.00 m above mean sea level) recorded a maximum average temperature of 30.74°C and a minimum of 19.21°C, with 859.10 mm of average rainfall and RH of 84.14% (Table S2). The experiment was laid out in an alpha lattice design with two replications, consisting of 20 blocks, accommodating 10 genotypes each. Each genotype (henceforth, all the genetic materials; reference set accessions and elite genotypes were called genotypes) was planted in two rows of 3-m length, with a row-to-row spacing of 90 cm and plant-to-plant spacing of 20 cm. The recommended package of practice was duly followed during the experimentation.

2.3 | Soil Sampling in Trial Locations

As per the USDA soil taxonomy, the soil of the three trial locations, namely, ICRISAT-Patancheru, RARS-Warangal and IIPR-Kanpur, belongs to sandy loam, vertisol and inceptisol, respectively. The soil samples were collected from a 30 cm depth in all three locations and were analysed for their mineral composition before crop sowing and after harvesting of the crop. This was mainly to ensure sufficient amounts of minerals in the soil and to accurately assess the performance of genotype (Table S3).

2.4 | Standard Operating Procedure (SOP) for Grain Micronutrients Estimation in Pigeonpea

The micronutrient estimation in grain requires a sophisticated operating procedure. Gathering a representative sample while reducing soil and dust contamination from harvesting or post-harvest processing equipment is critical in gaining credible results. As this work is the first of its kind in pigeonpea, standardizing the sampling procedure for grain micronutrient content: grain calcium content (GCaC), grain magnesium content (GMgC), grain iron content (GFeC) and grain zinc content (GZnC) estimation was a crucial step.

2.4.1 | Pre-Sowing Procedure

Knowing the soil nutrient status of the trial location was a pioneering step as it has a greater influence on grain micronutrient uptake. Accordingly, soil pH, soil texture and nutrient profiling were done prior to sowing. Based on substantial soil micronutrient composition, trialling was conducted at ICRISAT-Patancheru, RARS-Warangal and IIPR-Kanpur, respectively. Sowing was executed without any seed treatment and basal micronutrient application to have precision in grain micronutrient estimation without any external factors influencing the value.

2.4.2 | Crop Management

The genotypes were sown simultaneously across all three locations. Basal fertilizers, including diammonium phosphate (DAP), were applied at the recommended rate of 100 kg/ha. Pesticide applications were carried out as needed, while no micronutrient sprays were used throughout the trial. Irrigation was provided at critical stages of the crop and was harvested at maturity.

2.4.3 | Field Sampling

Because contamination from soil, dust, hands and equipment can impact micronutrient analysis, precautions were taken during harvesting. Randomly, five representative plants were selected per genotype in each replication. Fully matured pods were handpicked.

2.4.4 | Post-Harvest Procedure

The harvested pods were sun-dried until moisture content reached 12%. Then, they were subjected to manual threshing over machine threshing to avoid iron contamination and stored in clean muslin cloth bags. Furthermore, to prevent dust contamination, gloves were used to prevent direct hand contact while grain sampling.

2.4.5 | Grain Sampling

Micronutrient variability exists among plants of the same genotype and even within a single plant. Therefore, obtaining a representative working sample is crucial (Figure S1).

2.4.5.1 | Sample Collection Method.

1. Selected five individual plants from each genotype in both replications.
2. The grains were spread evenly on a clean, acid-washed tray.
3. The grain pile was flattened into a circular shape. The circle was divided into four equal parts.
4. Two diagonally opposite quarters were removed, and the remaining two were thoroughly mixed.
5. This quartering process was repeated until the desired sample size was obtained.
6. A 10-g working sample was then prepared for each genotype in both replications.

2.5 | Nitric Acid–Hydrogen Peroxide Digestion (Fe, Zn, Ca and Mg Analysis)

The wet digestion method involved weighing approximately 0.3 g of sample into 50 mL labelled polypropylene tubes. To each tube, 2 mL of nitric acid and 0.5 mL of hydrogen peroxide were added using a bottle-top dispenser. The tubes were vortexed for thorough mixing before being left to stand overnight at room temperature (20°C–22°C). The following day, the samples were vortexed again before undergoing digestion. The digestion process was conducted in a programmed digestion block, initially at 80°C for 30 min, followed by a gradual temperature increase to 125°C for 120 min. After digestion, the volume was adjusted to 25 mL with distilled water and mixed for 5 min using an orbital mixer or vortex. The samples were then filtered, and the supernatant was analysed using microwave plasma atomic emission spectroscopy (MP-AES) (Agilent) (Wheal et al. 2011).

2.6 | Micronutrient Estimation in Split Cotyledon and Coat

Consumption of pigeonpea is reported either with the seed coat (whole grain) or one devoid of the seed coat (dal/split cotyledon). Hence, understanding the micronutrients accumulation in dal as well as seed coat is pivotal. A small experiment was done using five cultivars to get whole grain to dal and whole grain to seed coat ratios. Accordingly, 100 g of whole grain of each cultivar was weighed, soaked in 1000 mL of water for 2 h and then oven-dried at 60°C for 7 h. The oven-dried samples were dehulled using a stone pestle–mortar (chakki) to get cotyledon and seed coat as separate entities. The separated cotyledon and seed coat were weighed to calculate the dehulling outturn. Simultaneously, whole grain samples weighing 15 g were soaked in 500 mL of water following the above-mentioned dehulling procedure. The separated cotyledon and seed coat of each cultivar were submitted to Charles Renard Analytical Laboratory, ICRISAT, to estimate micronutrients as per the methodology mentioned in Section 2.5.

The ratio of cotyledon to coat was measured in five elite pigeonpea cultivars. The average of these showed that 100 g of dry pigeonpea grains would include 15 g of coat and 85 g of cotyledon (Table S4). The percentage of micronutrients in each of the three components was estimated by the wet-lab method.

2.7 | Statistical Analysis

The Shapiro–Wilk test was used to establish the normal distribution of each dependent variable, and grain micronutrient data from all three locations were pooled. To assess the significance of the main and interaction effects of location and genotype variance components, a combined analysis of variance (ANOVA) across the locations was conducted, considering all factors—location, replication, block and genotype—as random. The SAS Mixed methods (SAS v9.4 SAS Institute Inc. 2023) were used to estimate and model the individual variances of locations to the error distribution using the REML (residual maximum likelihood) process. BLUPs (best linear unbiased predictions) were estimated for location, genotype and location × genotype from the combined ANOVA.

Broad-sense heritability for each micronutrient across locations was estimated and categorized into low (0%–30%), medium (30%–60%) and high (>60%) heritable (Johnson et al. 1955). Best linear unbiased predictions (BLUPs) (Schonfeld et al. 1986) were obtained for all micronutrients at each location and were used for all the downstream analysis. Data visualization and statistical analysis were conducted using 'R version 2024.04.2'. Violin plots illustrating the distribution of grain micronutrient contents at each location were generated using the 'ggplot2' package (Wickham et al. 2016). Correlation coefficients among agronomic traits and grain micronutrients were computed using the native R function 'cor ()' and visualized with the 'corrplot' package (Wei et al. 2021). The phenotypic distance matrix for the four micronutrients was constructed using Euclidean distance with the R package 'vegan'. A dendrogram was then generated using the Ward.D2 clustering method (Murtagh and Legendre 2014), and a heatmap depicting agronomic performance and grain micronutrient content of each genotype was created using 'heatmap3' (Zhao et al. 2021). The normal distribution of the data was checked using the 'Kolmogorov-Smirnov Normality Test' (Lilliefors 1967). Principal component analysis (PCA) was conducted using the 'factoextra' package in R. Finally, micronutrient-specific and micronutrient-dense genotypes were identified based on the *per se* performance and superiority over the trial mean.

3 | Results

3.1 | Variance Components of Grain Micronutrients in Pigeonpea

The variance components for grain micronutrients were analysed using pooled data from three locations. The results revealed that the variance components for genotype were significant for all four grain micronutrients: GCaC, GMgC, GFeC

TABLE 1 | Estimation of variance components for grain micronutrients among 200 pigeonpea genotypes evaluated across ICRISAT-Patancheru, RARS-Warangal and IIPR-Kanpur.

Trait	Components	Genotype	Location	Genotype × location	Location × replication	Location × replication × block
GCaC	Variance	67,020.00	8780.00	32,675.00	55.00	3957.00
	Standard error	9100.00	9228.00	5108.00	487.00	1769.00
	Z-statistic	7.36	0.95	6.40	0.11	2.24
	Prob. Chi-square	**<0.0001**	0.0162	**<0.0001**	0.9203	**0.0059**
GMgC	Variance	14,544.00	29,268.00	4727.00	210.00	910.00
	Standard error	1922.00	29,476.00	1106.00	287.00	409.00
	Z-statistic	7.57	0.99	4.27	0.73	2.22
	Prob. Chi-square	**<0.0001**	0.0011	**<0.0001**	0.1884	0.0057
GFeC	Variance	2.64	0.21	1.52	0.05	1.23
	Standard error	0.56	0.31	0.64	0.14	0.36
	Z-statistic	4.71	0.68	2.38	0.36	3.42
	Prob. Chi-square	**<0.0001**	0.32	0.01	0.66	**<0.0001**
GZnC	Variance	2.89	13.57	3.41	0.70	3.43
	Standard error	0.72	14.06	0.92	0.77	0.71
	Z-statistic	4.01	0.97	3.71	0.91	4.83
	Prob. Chi-square	**<0.0001**	0.0157	**<0.0001**	0.0419	**<0.0001**

Abbreviations: GCaC, grain calcium content; GFeC, grain iron content; GMgC, grain magnesium content; GZnC, grain zinc content; Prob. Chi-square, probability and chi-square.

and GZnC, with Z-statistics of 7.36, 7.57, 4.71 and 4.01, respectively, and chi-square probabilities of $p < 0.0001$ (Table 1). The genotype × location interaction was also significant ($p < 0.0001$) for GCaC, GMgC and GZnC, with Z-statistics of 6.40, 4.27 and 3.71, respectively. The percent contribution of variance components showed that genotype was the dominant factor for GCaC and GFeC, accounting for approximately 61.78% and 60.41% of the total variation, respectively (Figure 1). Additionally, the genotype × location interaction contributed 30.12% and 35% of the total variation for GCaC and GZnC, respectively, while location accounted for about 8.09% and 4.81% of the variation in GCaC and GFeC, respectively.

For GMgC and GZnC, the variance contribution analysis indicated that genotype accounted for 30% and 14.54% of the total variation, respectively. In contrast, location contributed the highest proportion, approximately 60% for GMgC and 68.29% for GZnC. Furthermore, the genotype × location interaction explained 10% and 17.16% of the total variation, respectively (Figure 1 and Table 1).

3.2 | Mean Performance and Genetic Variability Parameters

The comparison of mean values of 200 pigeonpea genotypes between three locations revealed a significant difference in the performance of the genotypes for all the grain micronutrients. The mean values and distribution of variation for GCaC, GMgC, GFeC and GZnC are depicted in the form of a violin plot (Figure 2 and Table S5). The GCaC among the genotypes ranged from 1047.00 ppm (ICP 9691) to 2506.30 ppm (ICP 1117), with an average of 1678.22 ppm. The GMgC ranged from 1327.04 ppm (ICP 1535) to 2106.90 ppm (ICP 1117), with an

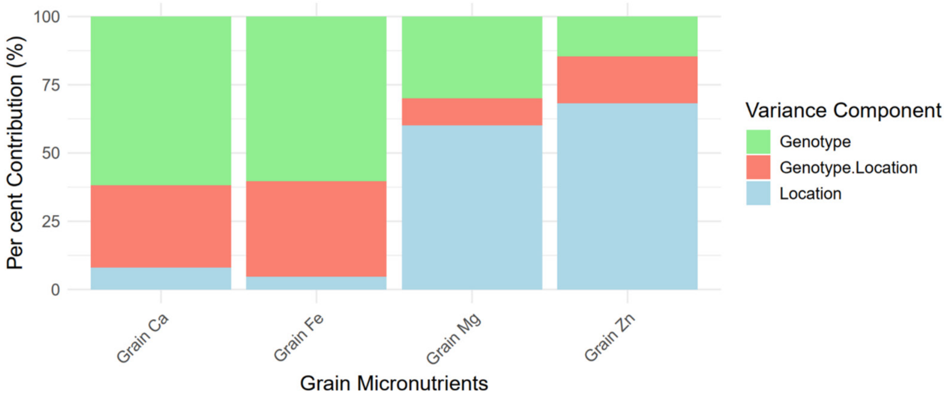

FIGURE 1 | Bar chart representing the percent contribution of variance components for grain calcium content (GCaC), grain iron content (GFeC), grain magnesium content (GMgC) and grain zinc content (GZnC) in pigeonpea.

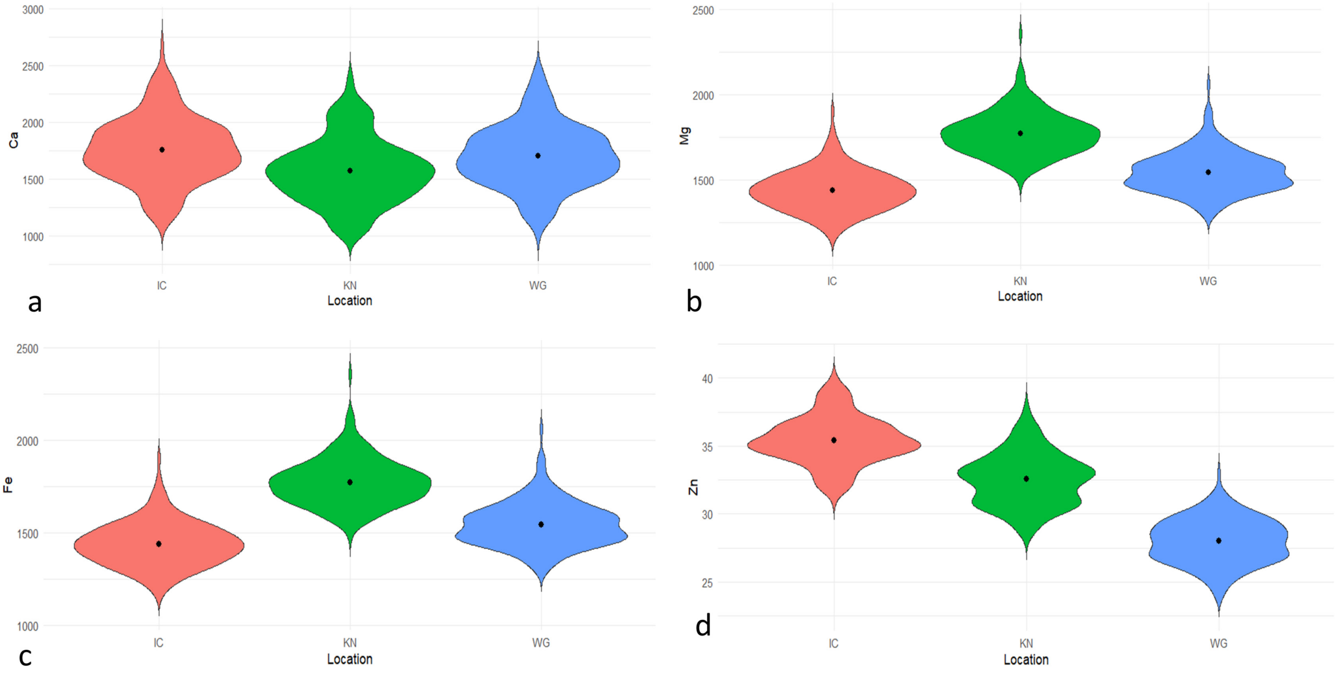

FIGURE 2 | Violin plots depicting the distribution of grain micronutrients—(a) grain calcium content (GCaC), (b) grain magnesium content (GMgC), (c) grain iron content (GFeC) and (d) grain zinc content (GZnC) across three locations (ICRISAT-Patancheru, RARS-Warangal and IIPR-Kanpur) for 200 pigeonpea genotypes. (*Note:* IC: ICRISAT-Patancheru, WG: RARS-Warangal and KN: IIPR-Kanpur).

average of 1585.45 ppm. The GFeC ranged from 27.64 ppm (ICP 7337) to 34.22 ppm (ICP 7269), with an average of 30.46 ppm. The GZnC ranged from 27.83 ppm (ICP 14832) to 36.10 ppm (ICP 4266), with an average of 31.99 ppm. Similarly, when comparing the performance of the individual genotypes to the trial mean for each micronutrient, it was observed that 100 genotypes exhibited higher GCaC than the trial mean (1678.22 ppm), while 96 genotypes showed higher GMgC than the trial mean (1585.45 ppm). High estimates of broad-sense heritability were observed for GCaC and GMgC, both at 76%, with genetic advance as percent of mean (GAM) values of 49.66% and 19.54%, respectively (Table 2). In comparison, GFeC had a heritability of 53.00% and a GAM of 19.30%, while GZnC showed a heritability of 46.00% and a GAM of 15.24% (Table 2). Based on the heritability and mean performance, the study identified ICP 1117 and ICP 15599 as micronutrient-dense genotypes, excelling in both GCaC and GMgC, while ICP 16180 and ICP 4266 emerged as the best-performing genotypes for GFeC and GZnC, respectively. These findings highlight the potential of donors for developing micronutrient-enriched pigeonpea.

3.3 | Correlation Among Micronutrients, Grain Yield-Related Traits

The correlation estimates based on pooled data among grain micronutrients and important yield-related traits (Figure 3) revealed significant relationships. GFeC and GZnC content exhibited a highly significant positive correlation ($r = 0.35$, $p < 0.01$). However, GFeC showed a significant but negative correlation with grain yield per plant ($r = -0.15$, $p < 0.05$) and days to 50% flowering ($r = -0.19$, $p < 0.05$). GCaC exhibited a strong and

TABLE 2 | Genetic variability for grain micronutrients in pigeonpea genotypes for pooled data.

		Range (ppm)		Genetic variability parameters				
Trait	**Mean**	**Minimum**	**Maximum**	**GCV**	**PCV**	**h^2 (bs) (%)**	**GAM (%)**	**CV%**
GCaC (ppm)	1678.22	1047.00	2506.30	15.43	17.68	76.00	49.66	14.71
GMgC (ppm)	1585.45	1327.04	2106.90	7.61	8.73	76.00	19.54	8.54
GFeC (ppm)	30.46	27.64	34.22	5.33	7.32	53.00	19.30	10.88
GZnC (ppm)	31.99	27.83	36.10	5.31	7.83	46.00	15.24	11.49

Abbreviations: GAM, genetic advance as percent of mean; GCV, genotypic coefficient of variation; h^2 (bs), broad sense heritability; PCV, phenotypic coefficient of variation.

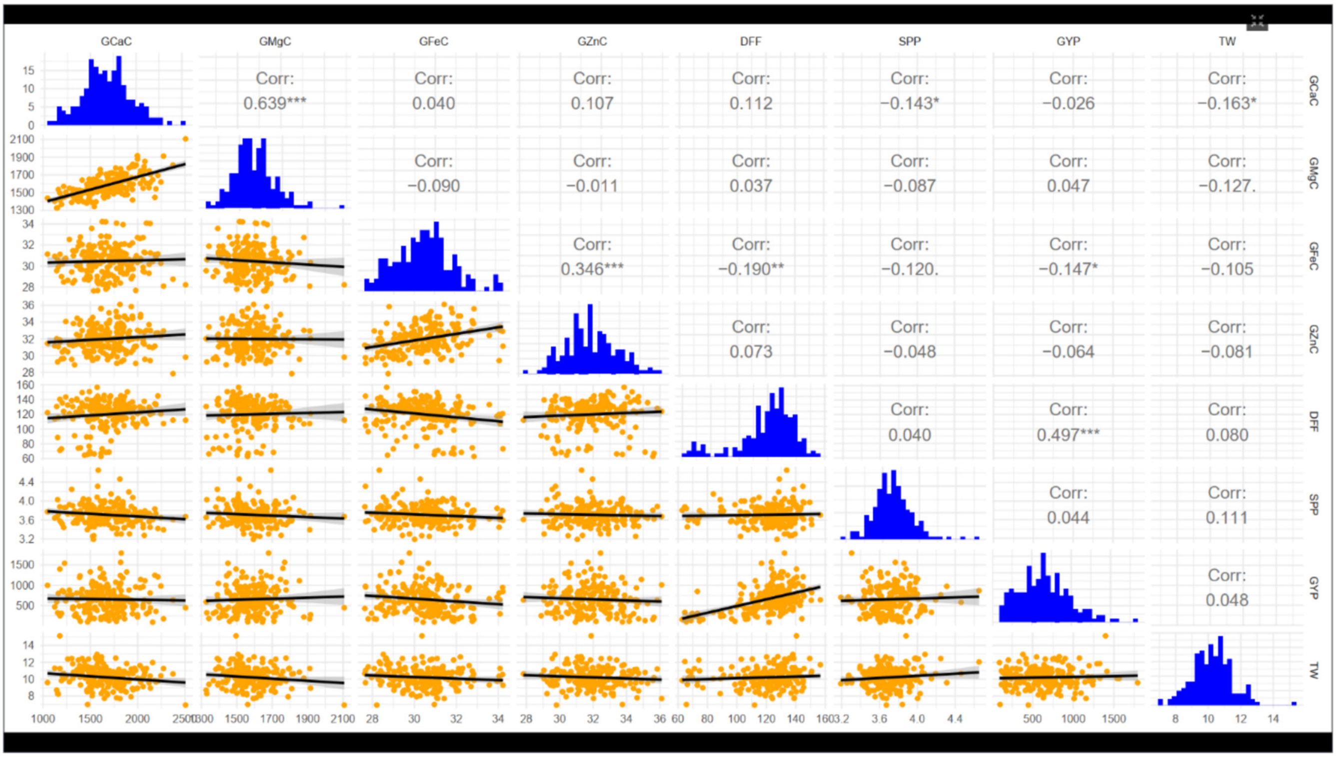

FIGURE 3 | Graphical representation of Pearson's correlation between grain micronutrients and agronomic traits pooled across three locations (ICRISAT-Patancheru, RARS-Warangal and IIPR-Kanpur). The analysed traits include days to 50% flowering (DFF), 100-seed weight (TW) and grain yield per plant (GYP), along with grain calcium content (GCaC), grain magnesium content (GMgC), grain iron content (GFeC) and grain zinc content (GZnC).

significant positive correlation with GMgC ($r = 0.63$, $p < 0.01$) and recorded a significant negative correlation with seeds per pod ($r = -0.14$, $p < 0.05$). On the other hand, the association of grain yield per plant with most of the micronutrients, namely, GZnC, GCaC and GMgC, was found to be non-significant.

3.4 | Grouping of Genotypes Based on Phenotypic Diversity

The clustering based on Euclidian distance matrix grouped the 200 genotypes into five distinct clusters (Table 3, Figure 4), with the number of genotypes in each cluster ranging from 13 to 72. Cluster II had the highest number of genotypes (72), followed by Clusters III and IV, each consisting of 43 genotypes. Cluster I included 29 genotypes, while Cluster V had the smallest group with 13 genotypes. The mean values of each cluster differed significantly for each micronutrient, highlighting distinct variations in grain micronutrients content. Cluster I exhibited a mean GCaC of 1621.86 ppm, GMgC of 1578.13 ppm, GFeC of 28.66 ppm and GZnC of 30.42 ppm. Cluster II showed GCaC and GMgC of 1542.98 and 1527.58 ppm, with GFeC and GZnC values of 30.64 and 32.16 ppm, respectively. Cluster III stood out with the highest mean GCaC (1910.13 ppm) and GMgC (1735.96 ppm), along with GFeC and GZnC values of 30.36 and 31.52 ppm. Cluster IV recorded the maximum mean values for GCaC and GMgC (1850.97 and 1586.49 ppm), while GFeC and GZnC were 31.65 and 33.68 ppm, respectively. Cluster V, characterized by lower GCaC (1208.36 ppm) and GMgC (1417.13 ppm), had mean GFeC and GZnC values of 30.08 and 30.57 ppm, respectively. The genetic similarity/dissimilarity among genotypes between and within clusters was determined by inter- and intra-cluster distances. It revealed that the average dissimilarity within each cluster ranged from $d = 0.11$ in Cluster V to $d = 0.33$ in Cluster IV (Table S6). This

TABLE 3 | Grouping of pigeonpea genotypes into clusters based on grain micronutrients profile.

Cluster no.	No. of genotype	Name of the genotype	Cluster mean (ppm)	
I	29	ICP 11281, ICP 11613, ICP 11833, ICP 12618, ICP 13571, ICP 14147, ICP 14444, ICP 14524, ICP 14832, ICP 15493, ICP 16264, ICP 16432, ICP 16674, ICP 6128, ICP 6668, ICP 6990, ICP 7035, ICP 7260, ICP 7314, ICP 7337, ICP 7803, ICP 7869, ICP 8384, ICP 8860, ICP 9045, ICPL 20092, ICPL 20202, ICPL 92016, TS3R	Grain Fe	28.66
			Grain Zn	30.42
			Grain Ca	1621.86
			Grain Mg	1578.13
II	72	GRG 152, ICP 10094, ICP 10276, ICP 10503, ICP 10613, ICP 10922, ICP 11230, ICP 11259, ICP 1126, ICP 1156, ICP 11633, ICP 11737, ICP 11754, ICP 11969, ICP 12105, ICP 12142, ICP 12410, ICP 12654, ICP 1279, ICP 13244, ICP 13575, ICP 13577, ICP 13998, ICP 14120, ICP 14294, ICP 14638, ICP 14770, ICP 14900, ICP 14903, ICP 14951, ICP 15068, ICP15382, ICP 16235, ICP 16309, ICP 2391, ICP 2405, ICP 2577, ICP 3451, ICP 348, ICP 4715, ICP 5142, ICP 5863, ICP 60, ICP 6049, ICP 6359, ICP 655, ICP 6739, ICP 6845, ICP 6859, ICP 6971, ICP 6992, ICP 7223, ICP 7413, ICP 7426, ICP 7798, ICP 7896, ICP 8146, ICP 8266, ICP 8757, ICP 8921, ICP 8941, ICP 8949, ICP 9049, ICP 9062, ICP 9414, ICP 9577, ICP 9750, ICPL 85063, ICPL 87119, ICPL 99050	Grain Fe	30.64
			Grain Zn	32.16
			Grain Ca	1542.98
			Grain Mg	1527.58
III	43	ICP 10447, ICP 10508, ICP 10531, ICP 10654, ICP 1071, ICP 1117, ICP 11321, ICP 11338, ICP 11627, ICP 11639, ICP 11890, ICP 11971, ICP 12123, ICP 12186, ICP 12515, ICP 13304, ICP 13662, ICP 14701, ICP 14840, ICP 15599, ICP 16440, ICP 2746, ICP 3046, ICP 3755, ICP 4029, ICP 4317, ICP 6815, ICP 6869, ICP 7, ICP 7028, ICP 7366, ICP 7420, ICP 7480, ICP 8144, ICP 8152, ICP 8242, ICP 8255, ICP 8618, ICP 8776, ICP 8793, ICP 9336, ICP 939	Grain Fe	30.36
			Grain Zn	31.52
			Grain Ca	1910.13
			Grain Mg	1735.96
IV	43	ICP 10559, ICP 10963, ICP 11320, ICP 11354, ICP 11406, ICP 11823, ICP 12680, ICP 12977, ICP 13195, ICP 13906, ICP 14209, ICP 14545, ICP 14722, ICP 14853, ICP 14868, ICP 14936, ICP 16180, ICP 16189, ICP 3049, ICP 4213, ICP 4266, ICP 4307, ICP 4903, ICP 6892, ICP 6929, ICP 7076, ICP 7130, ICP 7269, ICP 7487, ICP 7507, ICP 7952, ICP 8194, ICP 8211, ICP 8700, ICP 8817, ICP 8863, ICP 9236, ICP 9252, ICP 9891, ICP 995, ICPL 20201, ICPL 20203	Grain Fe	31.65
			Grain Zn	33.68
			Grain Ca	1850.97
			Grain Mg	1586.49
V	13	ICP 13431, ICP 14944, ICP 15185, ICP 1535, ICP 6123, ICP 7257, ICP 7266, ICP 9691, ICPL 19039, ICPL 19064, ICPL 20205, ICPL 87	Grain Fe	30.08
			Grain Zn	30.57
			Grain Ca	1208.36
			Grain Mg	1417.13

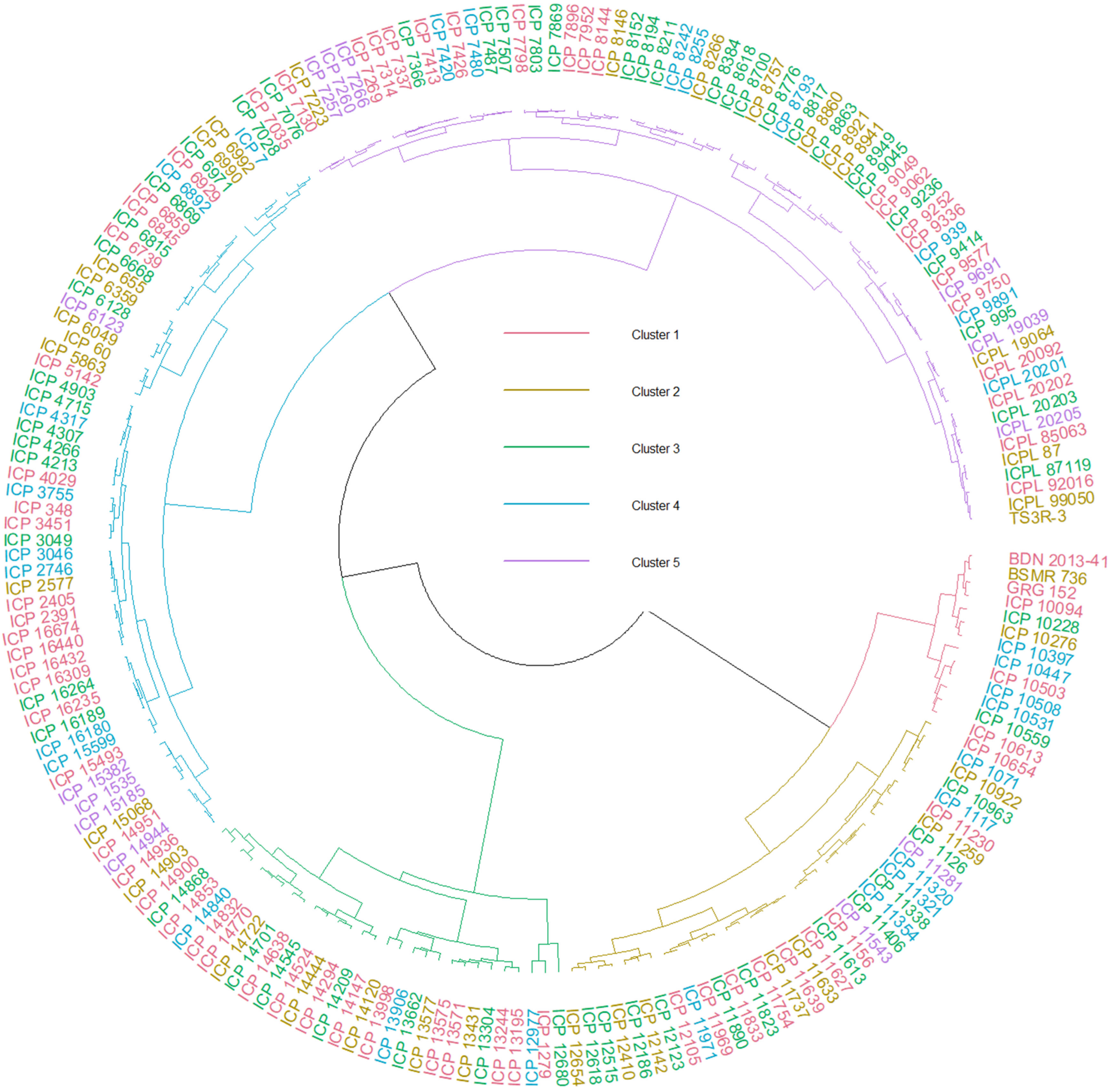

FIGURE 4 | Circular dendrogram representing hierarchical clustering of pigeonpea genotypes based on grain micronutrient content using Ward's D2 method, grouping genotypes based on their similarity in grain micronutrient content. Different colours represent distinct clusters, providing a clear visualization of genetic relationships. The outer ring labels correspond to genotype names, while the branches indicate their clustering patterns.

indicates that Cluster V was genetically uniform, while Cluster IV exhibited the highest variation within the cluster among the genotypes. The lowest inter-cluster distances revealed the degree of dissimilarity between the clusters. The lowest inter-cluster distance was observed between Clusters II and V ($d = 0.20$) (Table S6), suggesting a high degree of similarity between these two clusters. Conversely, the maximum inter-cluster distance was observed between Clusters IV and V ($d = 0.33$), indicating that these clusters are the most genetically diverse.

3.5 | Principal Component Analysis (PCA)

In this study, PCA was conducted to assess the genetic diversity of grain micronutrients. The PCA results effectively captured the extent of variation among the pigeonpea genotypes. The eigenvalues and the percentage of variation explained by the first four principal components (PCs) are presented in Table 4. The first PC (PC1) accounted for 41.16% of the total variance, while the second PC (PC2) explained 33.99% of the variance. Together, these two components captured 75.15% of the overall variation. In contrast, PC3 and PC4 explained comparatively lower proportions of the variation, at 16.20% and 8.63%, respectively. This suggested that the majority of genetic variation in grain micronutrients can be effectively represented by the first two components. Genotypes that are positioned closely together exhibited similar trait characteristics, while those farther apart exhibited more distinct profiles. The vector projections of GCaC, GMgC, GFeC and GZnC indicate their contribution to the PCs. Traits with longer vectors have a stronger influence on genotype differentiation (Figure 5a). Additionally, the correlation plot (Figure 5b) further supports these findings, highlighting strong associations between micronutrients. Notably, GCaC and GMgC exhibited a strong positive correlation, as indicated by the large, dark red circle. A similar, albeit weaker, positive association is observed between GFeC and GZnC, whereas other relationships such as GFeC with GCaC and GMgC and also GZnC with GCaC and GMgC appeared weaker or non-significant.

The trait loadings on the PCs highlight distinct patterns of variation. GFeC and GZnC were primarily associated with PC2, with loadings of 0.7060 and 0.6927, respectively, indicating a shared pattern of variation for these micronutrients. However, GZnC exhibited a negative loading on PC3 (−0.7090), whereas GFeC had a positive loading (0.7011), suggesting contrasting variation along this component. Conversely, GCaC and GMgC were strongly associated with PC1, with high positive loadings of 0.7081 and 0.6975, respectively, reflecting their common variation pattern. Interestingly, GCaC and GMgC exhibited opposite loadings in PC4, with GCaC having a positive loading (0.7025) and GMgC a negative loading (−0.7008), indicating their differential distribution along this component.

3.6 | Identification of Micronutrient Specific and Micronutrient Dense Genotypes

Genotypes with high micronutrient density were identified based on the superiority to the trial mean. The genotypes were categorized based on a single nutrient (nutrient-specific) and multiple nutrients (nutri-dense). For each trait, five superior genotypes were identified, and the yield attributing traits of those superior genotypes, along with grain micronutrient content, are also observed (Table 5). The top 5 micronutrient specific genotypes covered a range of 2119.96–2506.07 ppm for GCaC and 1820.10–2106.89 ppm for GMgC. Similarly, for GFeC and GZnC, the identified nutrient specific genotypes covered a range of 29.86–34.20 ppm and 35.26–36.10 ppm, respectively. The multi-micronutrient dense genotypes were identified from the top 5 nutrient specific genotypes for each trait. ICP 1117, ICP 15599 and ICP 7 were the genotypes rich in GCaC and GMgC. The results revealed an order of Ca > Mg > Zn > Fe micronutrient accumulation in whole grain.

TABLE 4 | Eigenvalues, contribution of variability and eigenvectors for the principal component axes in pigeonpea.

Trait	PC1	PC2	PC3	PC4
Eigenvalue	1.64	1.35	0.64	0.34
% Var. Exp.	41.16	33.99	16.20	8.63
Cum. Var. Exp.	41.16	75.15	91.36	100.00
GCaC	0.7081	0.0293	0.0640	0.7025
GMgC	0.6975	−0.1440	0.0400	−0.7008
GFeC	0.0058	0.7060	0.7011	−0.0993
GZnC	0.1090	0.6927	−0.7090	−0.0743

Abbreviations: % Var. Exp, percent of variance explained; Cum.Var. Exp, cumulative variance explained.

However, understanding the breakdown of whole grain to split cotyledon as well as seed coat is crucial to estimate micronutrients in consumable form. The result suggested that 100 g of whole grain pigeonpea splits into 85 g of cotyledon and 15 g of seed coat (Saxena et al. 2010). This whole grain to cotyledon ratio led to the calculation of the micronutrient retention in cotyledon and seed coat in pigeonpea.

Keeping this evidence, a panel of 10 (Table S7) genotypes representing a varying whole-grain micronutrient range was utilized for validation. The variability observed for iron content in the dal ranged from 2.57 to 3.53 mg/100 g, zinc content from 2.12 to 3.15 mg/100 g. While calcium content ranged from 37.84 to 120.33 mg/100 g and magnesium content ranged from 93.17 to 162.89 mg/100 g (Table S7). The iron, zinc and magnesium had no significant variation in seed coat. While calcium had shown slightly higher accumulation in seed coat than in cotyledon despite two genotypes deviating from the trend.

The distribution of micronutrients in whole grain, dal and seed coat was represented using box plots (Figure 6). Whole grain and dal exhibited no significant difference for iron, zinc and magnesium; however, the calcium content in whole grain varied significantly ($p < 0.05$) from dal and seed coat (Figure 7). The seed coat in pigeonpea tends to have slightly higher calcium content than the cotyledon (dal); however, this difference is

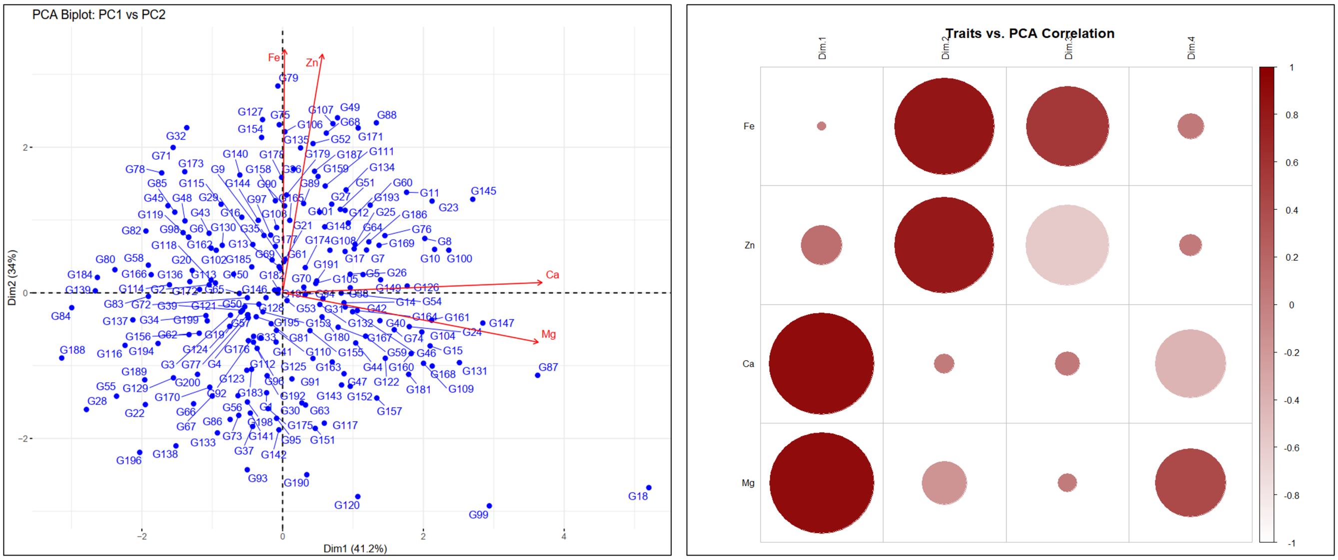

FIGURE 5 | Principal component analysis (PCA) of pigeonpea genotypes for grain micronutrients, grain iron and zinc, calcium and magnesium; (a) PCA biplot of genotypes and grain micronutrients (PC1 vs. PC2); (b) correlation of grain micronutrients with principal components.

TABLE 5 | Superior pigeonpea genotypes identified for grain micronutrients.

Grain calcium content

			Yield and its attributes		
Sl. no.	**Genotype**	**GCaC (ppm)**	**DFF (days)**	**GYP (g)**	**TW (g)**
1	ICP 1117	2506.27	112.00	82.60	6.93
2	ICP 15599	2273.17	107.00	38.00	11.26
3	ICP 2746	2373.20	112.00	60.20	10.61
4	ICP 10508	2213.05	145.00	781.70	9.76
5	ICP 11354	2119.96	126.00	31.00	9.91
6	Trial mean	1678.22	120.00	654.39	10.21

Grain magnesium content

			Yield and its attributes		
Sl. no.	**Genotype**	**GMgC (ppm)**	**DFF (days)**	**GYP (g)**	**TW (g)**
1	ICP 1117	2106.89	112.00	82.60	6.93
2	ICP 15599	1911.91	107.00	38.00	11.26
3	ICP 7480	1916.28	121.00	108.60	8.93
4	ICP 8793	1820.10	115.00	629.30	10.65
5	ICP 7	1853.15	112.00	236.70	10.83
6	Trial mean	1585.45	120.00	654.39	10.21

Grain iron content

Sl. no.	Genotype	GFeC (ppm)	DFF (days)	GYP (g)	TW (g)
1	ICP 7269	29.86	73.00	368.00	9.28
2	ICP 6929	34.20	121.00	108.70	10.55
3	ICP 16180	34.08	107.00	20.00	11.27
4	ICP 12680	34.07	117.00	432.80	9.85
5	ICP 9891	34.04	120.00	28.00	10.36
6	Trial mean	30.46	120.00	654.39	10.21

Grain zinc content

			Yield and its attributes		
Sl. no.	**Genotype**	**GZnC (ppm)**	**DFF (days)**	**GYP (g)**	**TW (g)**
1	ICP 4266	36.10	124.00	483.70	7.70
2	ICP 14868	35.84	128.00	55.00	11.20
3	ICP 14853	35.64	67.00	88.00	10.18
4	ICP 14936	35.58	63.00	42.50	9.85
5	ICP 11823	35.26	136.00	28.50	9.50
6	Trial mean	31.99	120.00	654.39	10.21

Abbreviations: DFF, days to 50% flowering; GYP, grain yield per plant; TW, test weight.

not statistically significant ($p > 0.05$). The character association study revealed that there is a significant positive association between grain micronutrients and dal micronutrients ($r = 0.97$ (iron), 0.96 (zinc), 0.94 (calcium) and 0.99 (magnesium); $p < 0.01$). Therefore, results revealed an order of Fe > Zn > Mg > Ca accumulation in the cotyledon.

4 | Discussion

Malnutrition and hidden hunger are the major global impediments affecting more than two billion people around the world (HarvestPlus 2025). It was reported that one in two children and two in three women showed micronutrient deficiency (Stevens

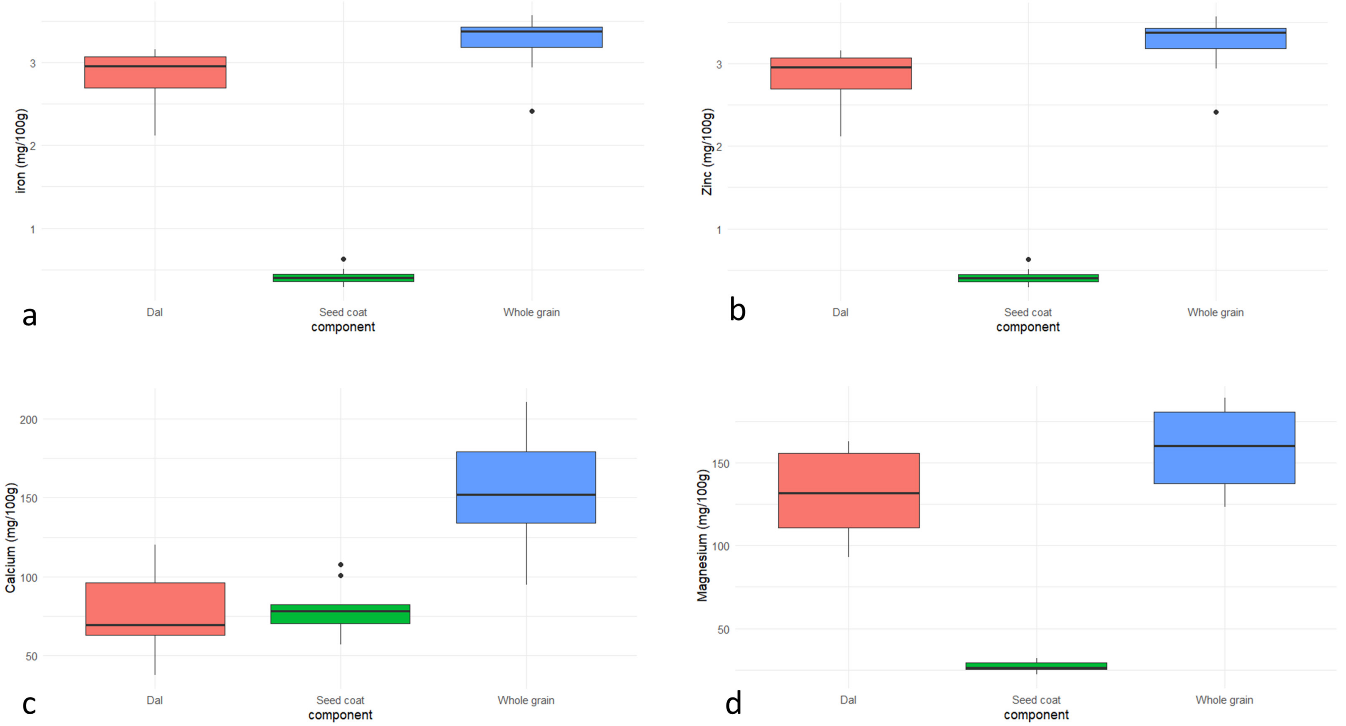

FIGURE 6 | Boxplot depicting the mean comparison of the micronutrient available per 100 g in whole grain (calculated), cotyledon and seed coat. (a) Iron, (b) zinc, (c) calcium and (d) magnesium.

et al. 2022). The consequences of micronutrient deficiencies are severe, leading to widespread health issues. Ca deficiency contributes to osteoporosis, resulting in weak bones and teeth, while inadequate Mg intake increases the risk of cardiovascular diseases. Additionally, deficiencies in other essential micronutrients, such as iron and zinc, cause anaemia and impaired growth, respectively (Kennedy et al. 2003). The availability of these important micronutrients through a staple diet is a sustainable means to alleviate malnutrition.

It is an advantage in pigeonpea as it finds its pivotal role in cereal-pulse based diet systems. Both the carbohydrate and protein requirements are fairly met by these daily dietary patterns. Enriching these crops for micronutrients adds another layer for delivering nutri-rich foods through regular staples instead of expensive micronutrient supplements, especially for vulnerable semi-arid folks. To enhance these micronutrients, developing a nutrient rich and agronomically robust genotype is a preliminary step. Understanding the genetic potential of these crops forms the follow-up for developing micronutrient-dense grains. In this regard, this study forms a base to initiate the biofortification breeding in pigeonpea.

The grain micronutrient content is highly dependent on soil micronutrient. The estimated micronutrient content in the subsurface soil layer (the upper 30 cm) in the experimental fields was above the critical limit. The pre-sowing soil profiling suggested a fair micronutrient distribution in the trial location, deciphering genotype's ability in consistent trait expression. The mixed model analysis indicated adequate genotypic variability for all four grain micronutrients. However, breeding for a qualitative trait is always challenged by the genotype × environment interaction (GEI). Whereas in the present study, the variance component attributable to the genotype × location interaction was found to be significant for GCaC, GMgC and GZnC, indicating the sensitivity of micronutrient accumulation to the environment. For GCaC, the majority of variation was attributed to genotype, suggesting a predominant genetic control and minimal environmental influence in grain calcium accumulation. This high genotypic variance indicates the feasibility of selecting for calcium-rich donors in pigeonpea. (Susmitha et al. 2022; Mashifane et al. 2024). GMgC exhibited a high variance contribution from both location and genotype × location interaction. However, the genotype effect remained substantial, indicating the presence of genetic variability that could be exploited in breeding programmes, which needs further validation from multi-season trialling.

For GFeC, the genotype component accounted for maximum variance, followed by location. The genotype × location interaction contributed minimally (Mashifane et al. 2024), suggesting that GFeC is largely controlled by genetic factors with lesser environmental influence. For GZnC, location emerged as a predominant factor contributing to variation, followed by genotype, with a relatively small genotype × location interaction effect. This suggested that GZnC in pigeonpea is heavily influenced by environmental conditions, further deriving evidence by testing multi-populations in multi-season (Susmitha et al. 2022; Chipeta et al. 2024; Gerrano et al. 2019).

Pigeonpea is found to be rich in calcium (Saxena et al. 2010). The current results of this study inferred that the GCaC in pigeonpea (104.70–250.06 mg/100 g) was found to be higher than major staple cereals (7.49–39.36 mg/100 g), such as rice, wheat, maize, pearl millet, sorghum and barley but lesser than Ca-dense finger millet (364 mg/100 g). Among grain legumes, soybeans have

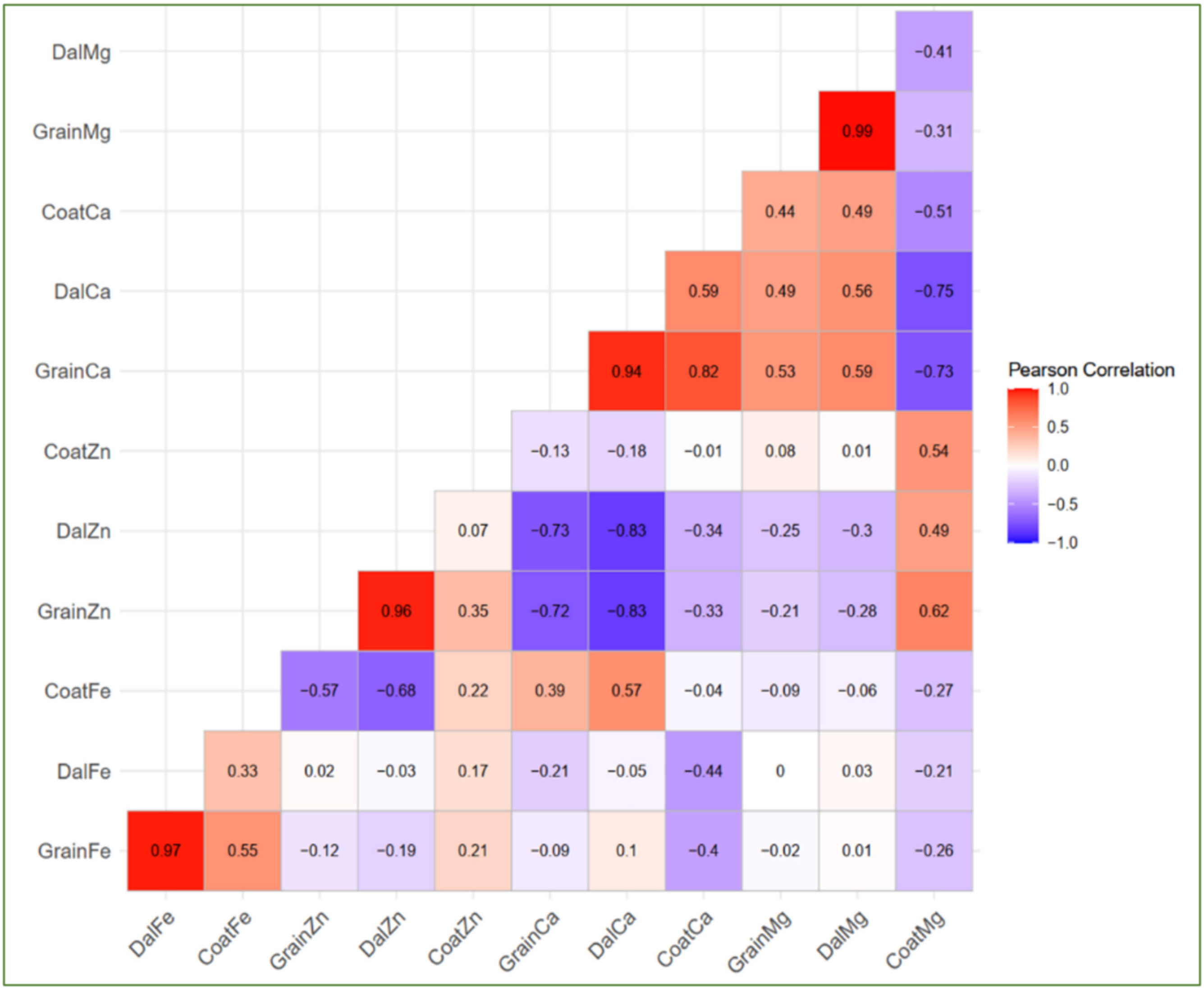

FIGURE 7 | Graphical depiction of Pearson's correlation among whole grain, dal and seed coat micronutrient contents in selected genotypes.

been known for having a high whole GCaC content (Longvah et al. 2017). However, our findings revealed that pigeonpea surpasses soybeans in whole GCaC, positioning it as the top grain legume. Further, the claim could be cross-validated by multiple seasons and varying soil types. The GFeC in pigeonpea (3.04 mg/100 g) is low when compared with other pulses like chickpea, black gram and horse gram (5.97–8.76 mg/100 g), while the GZnC (3.19 mg/100 g) is comparable with these pulses (2.71–3.37 mg/100 g; Gelaw et al. 2023). Interestingly, no notable variation was observed in the diversity panel (PI-GAP) for grain micronutrient content despite varying for maturity. These results offer an important insight for developing micronutrient-rich cultivars irrespective of maturity considerations.

The high heritability estimates observed for GCaC and GMgC suggested an efficient selection due to additive genes driving trait expression (Susmitha et al. 2022). However, variation for GFeC and GZnC in the current panel was found to be relatively low with moderate heritability inferring pre-dominate non-additive gene action governing the traits. This also concludes limited scope for genetic enhancements of these traits in pigeonpea. Instead, a niche breeding for nutri-pigeonpea emphasizing on high grain protein, Ca, Mg with optimum grain Fe and Zn would be a novel approach.

To substantiate this approach, a correlation study was undertaken between micronutrients and yield-attributing traits. It was observed that GCaC was positively correlated with GMgC and GFeC with GZnC, revealing a scope for simultaneous improvement for these traits. (Susmitha et al. 2022; Kennedy et al. 2003; Chipeta et al. 2024; Gerrano et al. 2019; Gelaw et al. 2023). On the contrary, the results revealed a negative correlation between GCaC and yield-attributing traits such as seeds per pod and test weight, which need further validation from multi-population screening across multi-seasons. A similar contradiction was observed for GZnC with per-plant yield, necessitating robust testing and validation before concluding the negative association of micronutrients with yield-attributing traits.

The clustering analysis of the 200 genotypes revealed a clear assemblage into five distinct clusters, each characterized by unique traits and variability. Notably, Cluster II was the largest, encompassing 71 genotypes, which suggested a high degree of similarity or shared characteristics among these genotypes. The genotypes that were grouped together may not be recommended for hybridization to create the variability for grain micronutrients. Clusters III and IV, with 43 genotypes each, also represent substantial groups of genetically as well as phenotypically similar genotypes for grain micronutrients. Henceforth, parental selection from these clusters should be based on inter-cluster distance to create trait variability. Clusters I and V, containing 29 and 13 genotypes, respectively, were the smallest groups identified. These variations in grain micronutrient content across the clusters provide critical insights for selecting potential donors and parents for the crossing programme. Similar results were reported by in previous studies as well (Susmitha et al. 2022; Mashifane et al. 2024; Gelaw et al. 2023; Kumar et al. 2020).

Further to breakdown the diversity among the genotypes, PCA was performed. In this analysis, eigenvalues greater than one were considered significant, and component loadings above ±0.3 were regarded as meaningful (Dhanushasree and Thanga Hemavathy 2022). The results revealed clear patterns of co-accumulating micronutrients (GCaC and GMgC; GFeC and GZnC). The first PC (PC1) explained 41.16% of the total variation, mainly influenced by GCaC and GMgC. The second PC (PC2) accounted for 33.99% of the variation, with GFeC and GZnC being the primary contributors. The PCA biplots categorized genotypes into four distinct quadrants, where smaller angles between vector lines indicated strong relationships among traits. The best-performing genotypes were positioned at the tips of these vectors. Our micronutrients PCA biplot showed that GFeC and GZnC had a positive association with PC2 and PC3 (Makebe and Shimelis 2023); nonetheless, GZnC displayed an inverse relationship in PC3. On the other hand, GCaC and Mg were dominant in PC1 and PC4, suggesting unique accumulation trends. Notably, GMgC had a strong positive loading in PC1 but a negative loading in PC4. The mapping of genotypes within the PCA quadrants depicted that those housed in the first quadrant were rich in GFeC and GZnC, while those in the second quadrant were higher in GCaC. Meanwhile, genotypes in the third quadrant were associated with elevated GMgC, highlighting the diverse distribution of micronutrients across the pigeonpea genotypes.

Based on the above findings, grain micronutrient content in pigeonpea exhibited high variability for GCaC and GMgC, whereas moderate variability for GFeC and GZnC. All four micronutrients showed high (GCaC and GMgC) to moderate (GFeC and GZnC) heritability. The identified micronutrient-specific and micronutrient-dense genotypes could be potential donors for introducing variability for grain micronutrient improvement. Thus, ICP 1117, ICP 15599, ICP 6929, ICP 9891 and ICP 4266 were identified as donors for all four grain micronutrients. The study concluded a micronutrient accumulation order of Ca > Mg > Zn > Fe in whole grain.

However, dal (split cotyledon) is a dominant consumption form globally. Understanding the micronutrient distribution in consumable form (dal) was pivotal as the seed coat is considered processing waste. Evidence supporting micronutrient loss in the seed coat was a necessity to derive into consumable quantity of cotyledon micronutrients. The findings showed that the amount of iron, zinc and magnesium retention in the dal is significantly higher than the seed coat, suggesting minimal loss during processing. While calcium deviates from this trend as it is a key structural component of the seed coat. The sturdiness of the seed coat is a result of calcium accumulation as a protective cover for embryonic cotyledons (Quilichini et al. 2022). Similar findings were evidenced in this study, where calcium accumulation in the seed coat was higher than in the cotyledon (Saxena et al. 2002). The study noted the pattern of micronutrient accumulation in the cotyledon varying from that of whole grain. The cotyledon had a micronutrient accumulation order of Fe > Zn > Mg > Ca compared with that of whole grain micronutrient accumulation order (Ca > Mg > Zn > Fe). The current study emphasized a selection for higher cotyledon micronutrient retention over whole-grain micronutrient. About 90% of Fe, 88% of Zn, 82% of Mg and 48% of Ca were retained in the cotyledon from whole grain. However, ICP 1117 (57%) and ICP 7 (60%) showed higher cotyledon calcium accumulation over the seed coat. Henceforth, direct selection of genotypes for higher cotyledon Fe, Zn, Mg and Ca is an efficient strategy for biofortification breeding in pigeonpea.

5 | Conclusions

This study lays a strong foundation for biofortification breeding in pigeonpea, providing crucial insights on whole grain as well as cotyledon micronutrient retention. The twofold variation in grain micronutrients, coupled with high heritability and strong positive correlation, presents a unique opportunity to enhance these essential micronutrients. A distinct micronutrient accumulation order of Fe > Zn > Mg > Ca was observed in cotyledon. Notably, ICP 1117 and ICP 7 emerged as superior genotypes for whole grain and cotyledon micronutrients, making them potential donors to establish the biofortification breeding. The development of high-yielding genotypes fortified with essential micronutrients is a powerful strategy for improving global food and nutritional security. Genetic studies for high cotyledon micronutrient accumulation are more efficient than those for whole grain micronutrient content. GWAS, candidate gene identification and multi-omics approaches would be a follow-up study to understand the underlying molecular mechanisms.

Author Contributions

Ashwini Kalyan: conceptualization, experimenting, SOP development, data collection, data analysis, validation, drafting the manuscript, review and editing. **Shruthi H. Belliappa:** conceptualization, methodology, validation, writing – review and editing. **Naresh Bomma:** trial management across three locations, conceptualization, methodology and review. **Muniswamy S. Sonnappa:** conceptualization, methodology, writing – review and editing. **Sateesh Naik:** trial management, conceptualization, writing – review and editing. **Sandhya Kishore:** trial management, review and editing. **Mahesh Pujar:** methodology, software, resources, writing – review and editing. **Praveen Kumar B.:** methodology, validation, writing – review and editing. **B. V. Tembhurne:** conceptualization, methodology, writing – review and editing. **Chinchole Laxuman:** conceptualization, methodology, writing – review and editing. **Sharanabasappa B. Yeri:** conceptualization, methodology and review. **Mallikarjun Kenganal:** writing – review and editing. **Krishna Gaiwal:** methodology, data analysis, writing – review and editing. **Sabbarigari Sai Vamshi:** methodology, writing – review and editing. **Anilkumar Vemula:** statistical analysis, data visualization and review. **Sean Mayes:** conceptualization, methodology and review. **Prakash I. Gangashetty:** validation, resources, funding acquisition, supervision, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Collecting a representative sample from five plants in pigeonpea. **Table S1:** List of 200 pigeonpea genotypes used in the study. **Table S2:** Weather parameters of all the trial locations during the 2023 rainy season. **Table S3:** Soil profiles pre-sowing and after harvesting of pigeonpea across three trial locations. **Table S4:** Partitioning of 100 g whole seeds into cotyledon and seed coat in elite pigeonpea cultivars. **Table S5:** Descriptive statistics for mean grain micronutrients evaluated in pigeonpea germplasm accessions grown in three trial locations. **Table S6:** Intra- and inter-cluster distances among pigeonpea genotypes. **Table S7:** Ten representative pigeonpea genotypes chosen from a PI-GAP for micronutrient profiling in dal and seed coat.