**Assessment of calcium accumulation in the wheat aleurone layer reveals phenotypic variation across two distinct environmental conditions**

**Abstract**

Calcium (Ca) is an essential macronutrient with key roles in plant physiology and human health, particularly in bone development and metabolic regulation. In wheat (Triticum aestivum L.), the aleurone layer serves as a nutrient-rich tissue, offering a promising target for calcium biofortification. This study assessed phenotypic variation in calcium accumulation within the aleurone layer across 137 diverse wheat genotypes grown under two contrasting agro-climatic environments: Meerut and Pantnagar. Calcium content was quantified using atomic absorption spectroscopy following di-acid digestion. Analysis of variance revealed significant genotypic variation (p < 0.001), while environmental and genotype × environment interactions were non-significant. Calcium concentrations ranged from 127.08 to 392.78 ppm in Meerut and 129.11 to 385.32 ppm in Pantnagar, with grand means of 261.44 ppm and 261.82 ppm, respectively. High estimates of heritability (98.53% in Meerut and 99.53% in Pantnagar) and genetic advance indicated strong genetic control over the trait. These findings highlight stable genotypes with elevated calcium levels that can serve as potential donors for biofortification. The data generated also establish a foundation for future QTL mapping and molecular dissection of calcium regulatory pathways in wheat. This work contributes to the broader goal of developing nutrient-rich wheat cultivars to alleviate dietary calcium deficiency through sustainable crop improvement strategies.

**Keywords:** phenotypic variation, environment, biofortification, G×E interaction

**1.Introduction**

Calcium is a fundamental structural and signaling element in plant biology, essential for maintaining cell wall integrity, membrane stability, enzyme activity, and mediating stress responses **[21,17].** In plant cells, calcium cross-links pectin in the cell wall, contributes to membrane stabilization, and acts as a key secondary messenger in diverse physiological processes such as root growth, gravitropism, and stress signaling **[4,9].** In addition to its vital roles in plants, calcium is also an indispensable mineral for human health. It plays critical roles in skeletal development, muscle contraction, nerve transmission, and intracellular signaling. Calcium deficiency is a widespread issue in low- and middle-income countries, where limited access to dairy and poor dietary diversity contribute to osteoporosis, rickets, and growth impairments **[5,2].**

Wheat, as a dietary staple for over 35% of the global population, represents a strategic vehicle for delivering essential micronutrients such as calcium through biofortification. However, the natural concentration of calcium in wheat grain is relatively low and largely restricted to the outer layers, especially the aleurone. The aleurone layer, a single-cell-thick outermost layer of the endosperm, is rich in minerals, enzymes, and phytochemicals and serves as a major reservoir of calcium, iron, zinc, and magnesium **[16,14]**.Though often removed during conventional milling, the aleurone layer contributes significantly to the nutritional quality of whole-grain wheat products. In addition to its nutritional value, the aleurone is biologically active, playing a role in enzyme release during germination and nutrient mobilization for the growing embryo. Calcium accumulation in the aleurone layer is known to vary substantially among wheat genotypes and is also highly influenced by environmental conditions such as soil pH, mineral content, temperature, and water availability[15. This variation is further modulated by genotype × environment interactions, making selection for high-calcium lines a complex but promising objective. Studies in wheat and other cereals have revealed that mineral concentrations in grains are quantitatively inherited and controlled by multiple small-effect loci, which can be mapped and utilized through genomic selection[**23**]. Furthermore, identifying and selecting stable, high-calcium genotypes suitable for cultivation in diverse agro-ecological zones can greatly enhance the impact of biofortification programs. In this context, the present study aims to evaluate the phenotypic variation in calcium concentration within the aleurone layer of a diverse panel of wheat genotypes grown under two contrasting environmental conditions. The insights generated will serve as a baseline for subsequent genetic analyses, such as QTL mapping or genome-wide association studies (GWAS), and contribute to the development of nutritionally enriched wheat varieties aligned with global calcium biofortification goals.

**2.Materials and Methods**

A total of 137 highly diverse and advanced wheat lines were obtained from the Department of Genetics and Plant Breeding, Chaudhary Charan Singh University, Meerut. These genotypes represented a wide range of growth habits and genetic backgrounds. The field evaluation of these lines was carried out under two distinct environmental conditions: at the experimental farm of CCSU, Meerut, and the Crop Research Centre at G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, during the Rabi season. Each genotype was sown in a plot consisting of five rows, each 2 meters long and spaced 20 cm apart, following a uniform layout at both locations.

At maturity, seeds were harvested from each plot and subjected to a standardized cleaning protocol to eliminate dust and other contaminants using deionized Milli-Q water. The cleaned seeds were then oven-dried at 70 °C for five hours to remove residual moisture. For aleurone layer isolation, seeds were soaked overnight in water, and the aleurone tissue was manually separated with care to ensure tissue integrity and consistency across genotypes.

For calcium estimation, the aleurone samples were digested using a microwave-assisted digestion system (Anton Paar, GmbH, Austria). Each sample was treated with 5 mL of a di-acid mixture composed of concentrated nitric acid and perchloric acid in a 3:1 ratio (Merck) and heated to 200 °C for 40 minutes until a clear solution was obtained. Digests were transferred to Falcon tubes, and the volume was adjusted to 20 mL with Milli-Q water prior to analysis. Calcium content was quantified using an atomic absorption spectrometer (AA240FS, Agilent Technologies, CA, USA). To ensure data reliability and precision, multiple quality control measures were adopted. These included regular performance checks of the instrument through calibration with certified standard solutions, blank runs, and verification of sensitivity. Additionally, certified reference materials (CRMs) were used to validate analytical accuracy, and where applicable, duplicate sample analyses were performed to assess repeatability and minimize measurement uncertainty **[11].**

The phenotypic data collected from both environments were subjected to statistical analysis to determine the variation in calcium concentration across genotypes. Analysis of variance (ANOVA) was performed using the Agricolae package in R (R Core Team, 2016) to evaluate the significance of genotype effects under each environment. F-tests were conducted at significance thresholds of 0.05, 0.01, and 0.001. Descriptive statistics, including mean, coefficient of variation (CV), and standard error of the mean (SEM), were calculated using the Pastecs package in R to summarize the distribution and variability of calcium concentration in the aleurone layer across the evaluated genotypes

**3.Results**

A wide range of variation was observed in calcium concentration in the aleurone layer of 137 wheat genotypes evaluated under two environments. Analysis of variance showed that the effect of genotype was highly significant (p < 0.001), indicating substantial differences among the tested lines (**Table 1**). In contrast, the effects of environment and genotype × environment interaction were non-significant (p > 0.05), suggesting a relatively stable expression of the trait across both test locations. Replication also had no significant effect (**Table 1**).

**Table 1: Analysis of variance (ANOVA) for 137 wheat genotypes calcium concentration in aleurone layer**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Df** | **Sum Sq** | **Mean Sq** | **F value** | **Pr(>F)** |
| **Genotypes** | 137 | 1567379 | 11440.7 | 408.5987 | <2e-16 \*\*\* |
| **Replication** | 1 | 19 | 18.9 | 0.676 | 0.4117 |
| **Environment** | 1 | 20 | 19.9 | 0.7108 | 0.3999 |
| **Genotype:Environment** | 136 | 3799 | 27.9 | 0.9977 | 0.4995 |

**Significant at \*\*\*0.001.**

Descriptive statistics indicated that calcium concentration in the aleurone layer ranged from 127.08 ppm to 392.78 ppm in Environment 1 (Meerut), with a grand mean of 261.44 ppm. In Environment 2 (Pantnagar), calcium levels ranged from 129.11 ppm to 385.32 ppm, with a grand mean of 261.82 ppm (**Table 2**). Standard error of mean was 4.60 in Environment 1 and 2.63 in Environment 2. The critical differences (CD) at 5% and 1% levels were 12.86 ppm and 16.99 ppm in Environment 1, and 7.34 ppm and 9.70 ppm in Environment 2, respectively.

The estimated genotypic variance was 2838.10 and 2888.95 in Environment 1 and 2, respectively, while phenotypic variance was recorded as 2880.41 and 2902.73. Environmental variances were relatively low, with values of 42.32 and 13.78 across the two environments. The phenotypic coefficient of variation (PCV) was 20.53% in Environment 1 and 20.58% in Environment 2, closely matching the genotypic coefficient of variation (GCV), which was 20.38% and 20.53%, respectively. Environmental coefficient of variation was 2.49% and 1.42% (**Table 2**). Heritability in the broad sense was high, estimated at 98.53% for Environment 1 and 99.53% for Environment 2. The genetic advance was calculated as 108.93 ppm and 110.46 ppm in the two environments, corresponding to 41.67% and 42.19% of the respective means (**Table 2**).

**Table 2: Descriptive statistics of 137 hexaploid wheat genotypes.**

|  |  |  |
| --- | --- | --- |
| **Descriptive statistics** | **Environment 1 (E1)** | **Environment 2 (E2)** |
| Maximum | 392.7825 | 385.3233 |
| Minimum | 127.0798 | 129.1133 |
| Grand Mean | 261.4429 | 261.8245 |
| Standard Error of Mean (SEm) | 4.5999 | 2.6251 |
| Critical Difference (CD) 5% | 12.8644 | 7.342 |
| Critical Difference (CD) 1% | 16.9946 | 9.6996 |
| Environmental Variance | 42.3177 | 13.7821 |
| Genotypic Variance | 2838.0966 | 2888.9514 |
| Phenotypic Variance | 2880.4143 | 2902.7335 |
| Environmental Coefficient of Variance | 2.4882 | 1.4179 |
| Genotypic Coefficient of Variance | 20.3768 | 20.5286 |
| Phenotypic Coefficient of Variance | 20.5282 | 20.5775 |
| Heritability (Broad Sense) | 0.9853 | 0.9953 |
| Genetic Advance | 108.9349 | 110.4597 |
| Genetic Advance as percentage of mean | 41.6668 | 42.1885 |

**4.Discussion-**

This study demonstrated considerable phenotypic variability in calcium accumulation within the aleurone layer of wheat grain across two contrasting environments. The analysis of variance revealed significant genotypic effects and high heritability, suggesting that calcium concentration in the aleurone layer is largely governed by genetic factors and can be effectively improved through selection. The non-significant genotype × environment interaction further reinforces the stability of genotypic performance across locations, indicating that certain genotypes express consistently high calcium levels regardless of environmental variation. This finding aligns with previous reports, where calcium and other micronutrient concentrations in wheat and related cereals exhibited stable inheritance patterns and were amenable to genetic enhancement **[20,6].** The broad range of calcium concentration observed in this study supports the feasibility of selecting superior lines with enhanced mineral density, which could serve as valuable donors in wheat biofortification programs.

The aleurone layer of wheat is recognized as a nutrient-rich tissue that plays a vital role in seed physiology and nutritional value. Despite its removal during conventional milling, it remains a critical focus for biofortification due to its enrichment in minerals such as calcium, zinc, iron, and magnesium. Calcium, in particular, is not only essential for seed structure and viability but is also of significant dietary importance in human nutrition. As a macronutrient, calcium regulates bone development, vascular function, and metabolic signaling. In plants, calcium is involved in various cellular processes, including cell wall formation, enzyme activation, and response to biotic and abiotic stressors **[21,16**]. Enhancing calcium content in the aleurone layer, therefore, contributes to both plant health and human dietary quality, especially in regions where dairy intake is limited and cereal-based diets predominate.

At the molecular level, calcium transport and homeostasis are mediated by a network of specialized transporters, sensors, and regulatory proteins. Among these, the cation/H⁺ exchanger (CAX) gene family plays a central role in vacuolar calcium sequestration. The wheat gene TaCAX1, an ortholog of Arabidopsis CAX1, encodes a vacuolar antiporter that removes excess cytosolic calcium by actively transporting it into the vacuole, thereby maintaining calcium homeostasis during seed development. Overexpression of CAX genes in rice and other cereals has led to enhanced grain calcium content, providing a proof-of-concept for their potential utility in wheat [**9,3**]. In addition to CAX transporters, calcium ATPases such as TaACA4 and TaECA3 contribute to calcium mobilization across cellular membranes. TaACA4 is typically associated with calcium efflux at the plasma membrane, while TaECA3 facilitates internal compartmentalization within the endoplasmic reticulum. These ATPases are highly active during grain filling and may contribute significantly to calcium loading in the aleurone tissue **[1,15]**.

Calcium-binding proteins and signaling molecules further modulate calcium distribution and utilization within developing seeds. Annexins such as TaANN6 are calcium-dependent membrane-associated proteins involved in vesicular trafficking and membrane repair, and are thought to regulate calcium flux during stress and grain maturation **[11]**. Calmodulins (CaM) and calmodulin-like proteins (CMLs), including TaCaM1 and TaCML39, act as calcium sensors that interpret calcium signals and trigger downstream regulatory pathways affecting nutrient mobilization and grain development **[12].** These sensor proteins form a signaling cascade with other components to coordinate calcium storage and redistribution during grain maturation. Additionally, broad-spectrum metal transporters such as TaNRAMP2 and TaZIP5, traditionally associated with iron and zinc transport, may also contribute to calcium distribution due to their ability to transport multiple divalent cations. Their expression in developing grains suggests a potential indirect role in modulating calcium concentration within grain tissues, including the aleurone layer **[18**]. Beyond transporters and sensors, transcriptional regulators play key roles in controlling mineral allocation. Transcription factors such as TaNAC29 and TabZIP60 have been implicated in nutrient remobilization and stress responses during grain development. TaNAC29 is an ortholog of AtNAC involved in senescence and nutrient recycling, while TabZIP60 regulates a suite of stress-responsive genes and may influence the expression of calcium-related transporters **[19,7].**

The complex interplay among these genetic components underscores the multifaceted regulation of calcium accumulation in wheat grain. Advances in wheat genomics, transcriptomics, and phenomics provide unprecedented opportunities to dissect these pathways further. Integrating the high phenotypic variation observed in this study with genomic analyses, such as QTL mapping and genome-wide association studies (GWAS), will facilitate the identification of key alleles and their deployment in biofortified wheat breeding pipelines. The combination of stable phenotypic performance, high heritability, and emerging molecular targets presents a promising platform for developing wheat varieties with enhanced calcium content, focused specifically on the aleurone layer—a key reservoir of nutritional value in the grain.

**5.Conclusion**

The present investigation revealed significant genetic variability in calcium accumulation within the aleurone layer of wheat grain across diverse genotypes and contrasting environments. The high heritability and genetic advance estimates indicate that calcium content is a stable and heritable trait, largely unaffected by environmental fluctuations. The identification of high-calcium genotypes, combined with insights into calcium transport and regulatory mechanisms from recent molecular studies, provides a strong foundation for targeted biofortification. These findings support the potential of using the aleurone layer as a strategic focus for improving grain calcium content in wheat breeding programs. Future integration of phenotypic data with genomic tools such as QTL mapping and marker-assisted selection will be essential for developing nutritionally enriched wheat cultivars to address calcium deficiency in human diets, particularly in regions where cereal-based foods form a dietary staple.

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