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Comparison of Zinc Fertilizer Application Response in Biofortified and Non-Biofortified Wheat (*Triticum aestivum* L.) Varieties and Advanced Lines

Zaheer Ahmed^{1,2} , Ayesha¹, Aroma Afzal², Mushood Ali², Muhammad Hassaan Khan², Raheela Rehman^{*1,2}

Abstract

Wheat (*Triticum aestivum* L.) is a global staple crop, yet it frequently exhibits zinc (Zn) deficiency, especially in calcareous soils. This study evaluated the effects of soil, foliar, and combined Zn applications on agronomic traits and grain Zn content across ten wheat genotypes, including biofortified and non-biofortified lines. The experiment, conducted under a split-plot RCBD, included four treatments: control (T0), soil application (T1), foliar application (T2), and combined soil + foliar (T3) using ZnSO₄·7H₂O. Significant improvements ($p < 0.01$) were observed in plant height, flag leaf area, mother spike weight, thousand-grain weight (TGW), and grain yield per plant (GYPP) under Zn treatments. Akbar-19 (G3) and SD-75 (G9) were the top-performing genotypes under T3, with GYPP increasing up to 98.43 g plant⁻¹ and TGW reaching 33.27 g. Grain Zn content also surged under T2 and T3, with peak values of 519.76 ppm and 776.58 ppm, respectively, compared to control. Principal component analysis revealed that the first three components PC1 (25.5%), PC2 (17.9%), and PC3 (15.4%) explained 58.8% of the total variance, with PC1 driven mainly by yield-related traits. Correlation analysis revealed strong positive associations between GYPP and TGW ($r = 0.82$), spike weight ($r = 0.78$), and plant height ($r = 0.74$). These findings indicate that integrated soil and foliar Zn application can considerably enhance wheat yields and grain Zn content, especially in genotypes Akbar-19 and SD-75, offering a sustainable approach to mitigating Zn deficiency in human diets.

Keywords: Biofortification, Wheat, Zinc content, Foliar zinc, Soil and foliar zinc.

¹Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad 38040, Pakistan; ²Center for Advanced Studies in Agriculture and Food Security (CAS-AFS), University of Agriculture, Faisalabad 38040, Pakistan

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Introduction

Wheat is the main source of micronutrients worldwide. It is a vital part of a balanced diet, making up about one-fifth of daily protein intake (Pervin et al., 2025; Yang et al., 2011). Hexaploid wheat, which has six sets of chromosomes, belongs to the Poaceae (Gramineae) family, can self-pollinate, and is widely grown (Venske et al., 2019). The three main types of wheat cultivated are hexaploid (common wheat, *T. aestivum*), tetraploid (emmer wheat), and diploid (einkorn wheat) (Zhang et al., 2016). Wheat plays a key role in food security by providing essential nutrients to people in developing countries who depend on plant-based diets for various reasons (Nadim et al., 2012). Agriculture is a vital sector in Pakistan, making up 24% of the country's GDP, highlighting its significant economic contribution (Government of Pakistan, 2024). Pakistan is a leading wheat producer, contributing roughly 3.5% of the world's total yield (Ashraf et al., 2020). However, by 2050, global wheat demand is expected to rise sharply, requiring farmers to boost their annual production (Abbas et al., 2009; Guarin et al., 2022). Variations in micronutrient levels, such as zinc and iron, can cause major health and nutritional problems, leading to malnutrition (Asgari et al., 2022). Malnutrition affects millions due to zinc deficiencies in diets (Ahmad et al., 2012; Bhutta, 2011). Pakistan exhibits high rates of nutrition-related deficiencies among children under five and mothers (Khattak et al., 2017). Zinc is a critical cofactor for over 100 enzymes and is essential for DNA and RNA synthesis, immune function, and hormone production. A diet lacking zinc can hinder children's growth and overall health (Bhutta & Hanif, 2010; Hussain et al., 2022; Wu et al., 2019).

Usually, crops grown in calcareous soil show a zinc deficiency. In Pakistan, the Potohar Plateau region, 80% of the sampled wheat fields lacked Zn (Rafique et al., 2006). Since alkaline and calcareous soils are widespread in Pakistan, with a pH above 7 and very little organic matter, they often limit zinc availability, leading to poor absorption by plants (Maqbool et al., 2015; Premalatha et al., 2023). Similarly, half of the world's grain-producing lands are low in zinc, causing zinc-deficient malnutrition globally (Das & Green, 2016). Recent data shows that high-yielding wheat varieties contain less zinc, contributing to ongoing zinc deficiency among about one billion people worldwide (Hao et al., 2014; Sánchez-Palacios et al., 2023). Another reason for poor Zn uptake is that modern breeding practices, which reduce genetic diversity, lead to lower zinc content in wheat grains (Niu et al., 2023). Experts are exploring various ways to address this issue. One solution is foliar zinc application, which has been reported to significantly improve grain yield and zinc absorption (Sánchez-Palacios et al., 2023). Another approach involves the use of *Bacillus* spp., Zn fertilizers, and Nano-Zn particles, which have shown positive results in zinc uptake, crop yields, and nutrient status (Yadav et al., 2023; Upadhyay et al., 2023).

In developing countries, to address zinc deficiency, several methods are implemented, including consuming supplements, adding minerals to foods, eating a varied diet, and increasing zinc content in foods. Enhancing staple crops with nutrients (also known as biofortification) is becoming a more reliable, sustainable, and cost-effective approach (Kour et al., 2020). Using zinc fertilizers such as $ZnSO_4 \cdot 7H_2O$ or chelate-type fertilizers increases the amount of available zinc in soils under alkaline conditions (Gregory et al., 2017). Despite these advantages, economically sound farmers may avoid using them because synthetic chelates are becoming expensive and scarce (Ullah et al., 2022). More environmentally friendly and financially sustainable options include farmyard manure, poultry litter, green manure, and sewage sludge (Thapa et al., 2021). Since these materials chelate zinc, they enhance the likelihood that plants will absorb and utilize zinc properly

(Ullah et al., 2022). Additionally, using natural materials in the soil improves soil structure, boosts beneficial microbial populations, and promotes more efficient zinc recycling (Almås et al., 2000; Laurent et al., 2020). With the recent growth of the poultry industry in the country, their litter provides surplus organic matter that helps improve soil fertility (Laurent et al., 2020). Growing crops using both types of nutrients might be the most effective and safest way to increase yields and enhance zinc levels in crops while maintaining healthy soil (Gregory et al., 2017).

Pakistan is an example, where cereals fulfil the sole dietary requirements of the people, causing nutrient shortages as well (Kour et al., 2020). The objectives of this study is to evaluate the response Zn in soil and foliar spray on morphological traits and to profile the micronutrients of wheat germplasm to check the Zn content and localization of Zn in the selected wheat genotypes for wheat grain.

Materials and Methods

Plant material

Ten different varieties/genotypes, including three high-Zn biofortified (Zincol-16, Akbar-19, Nawab-21), five traditional (non-biofortified - Johar-19, Fakhra-e-Bakhar, Anaj-17, Bakhar Star, Ghazi-19) and two synthetics (SD-166 and SD-75), were chosen. The seeds were obtained from the Wheat Genetics Laboratory (Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad) and sown on experimental farm (at the university) in the period between 2022 and 2023. Standard methods were used to determine the soil's pH, EC, texture, organic matter, calcium carbonate content, and the amount of available Zn.

Experimental Design

The experiment was carried out in three replicates using the split-plot design under RCBD with 15 cm P-P space and 30 cm R-R space. A total four treatments: T0 (without Zn), T1 (Zn given to soil at tillering), T2 (Zn sprayed on foliage at crop maturity), and T3 (Zn given to both soil and foliage). At the beginning of the season, basal fertilizers (containing 130 kg N, 90 kg P₂O₅, and 60 kg K₂O) were applied to the soil, while half of the urea was applied at sowing time, and the other half was divided into two more splits. In T1 and T3, Zn was given as ZnSO₄·7H₂O at 50 kg/acre in the soil, whereas T2 received a foliar spray (0.4% ZnSO₄·7H₂O and 0.02% Tween-20) in the evening. The grains were harvested at 14% moisture, threshed, cleaned and dried before analysis.

Physio-morphological Analysis

Factors that were measured included traits plant height (PH), peduncle length (PL), flag leaf area (FLA), spikelets per spike (SPS), no. of tillers per plant (NTPP), spike length (SL), mother spike weight (MSW), thousand grain weight (TGW), grain yield per plant (GYPP).

Micronutrient Assessment

Zinc content was assessed by using the different levels of zinc treatment. The analysis of micronutrients for all genotypes were carried out in the Wheat Biotechnology Lab at CAS-AFS in June 2023 using Atomic Absorption Spectrophotometer (AAS). Seed samples of all genotypes were ground using an electronic grinder. Samples from each genotype (about 0.5 g) were transferred into a separate labelled flask (covered in aluminium foil) and mix it with 3.5 ml of di-acid mixture (perchloric acid and nitric acid in a 2:4 ratio, respectively) solution for overnight. Later, those flasks were heated on a hotplate until a clear transparent solution, diluted with 50 mL distilled water and filtered into separately labelled plastic bottles. The zinc content was measured as parts per million (ppm) by using atomic absorption spectrophotometer (AAS).

Microscopic Screening

The Zn was labelled and visualized using a dithizone staining method. Grains were soak in water and then coloured with a diphenyl thiocarbazone solution. Microscope images were taken to analyze how Zn was distributed in the material.

Statistical Analysis

Statistical analysis was done by applying ANOVA (Steel et al., 1997). Furthermore, the traits that showed significant ($p < 0.05$) differences were analyzed by correlation and principal components analysis (Kwon & Torrie, 1964) to understand which traits are more closely related and selection of top-performing genotypes through bi-plot and component loadings.

Results

Physio-morphological Parameters

In this study, ANOVA (Table 1) suggested that plant height, peduncle length, flag leaf area, number of spikelets, number of tillers, spike length, mother spike weight, 1000-grain weight, grain yield per plant, and grain zinc content were significant factors. This indicated that there were significant differences between treatments and genotypes. The results showed that wheat genotypes exhibited a wide range of genetic variation and responded differently to zinc (Zn) application. The interaction between treatment and genotype ($B \times C$) for all traits indicated that some genotypes performed better under specific treatment methods. Foliar treatments had a greater impact on genotypes Akbar-19 and SD-75. Plant height (63.15 cm) and 1000-grain weight (33.2655 g) showed the highest mean square values among treatments and genotypes, indicating they were strongly affected by Zn application and genetic control. From these results, Zn application appeared to influence agronomic traits significantly, and genotypes Akbar-19 and SD-75 demonstrated superior Zn uptake, supporting their inclusion in biofortification programs.

Table 1. ANOVA for Split-RCBD of all the traits

Source	Replication (A)	Treatment (B)	Error A × B	Genotypes (C)	B × C	Error A × B × C
	DF=2	DF=3	DF=6	DF=9	DF=27	DF=72
PH	1.327	195.399**	0.386	85.35**	63.158**	1.89
PL	6.2674	32.6859**	3.0901	32.096**	15.5328**	0.6984
FLA	9.833	147.701**	0.832	71.949**	14.971**	1.922
NSP	0.6043	7.051*	1.431	17.1067**	1.2446**	0.3466
NTP	2.8516	14.4362**	0.1375	24.2393**	9.09**	0.9753
SL	5.0325	23.9428**	1.6797	4.7763**	0.5603**	0.1729
MSW	0.00169	1.59141**	0.00858	0.38358**	0.09623**	0.0073
TGW	0.01356	1042.83**	0.00422	66.3826**	33.2655**	0.0063
GYP	14.003	45.3422**	1.1595	98.4314**	2.8766**	0.4759
GZC	0.537	776.580**	0.924	519.761**	28.952**	0.936

$P \leq 0.01$ highly significant**, $P \leq 0.05$ significant*, $P > 0.05$ non-significant: Where SOV=source of variation, DF=degree of freedom. PH (plant height), PL (peduncle length), FLA (flag leaf area), SPS (spikelets per spike), NTPP (no. of tillers per plant), SL (spike length), MSW (mother spike weight), TGW (thousand grain weight), GYPP (grain yield per plant), GZC (grain Zn content)

This graphical representation (Figure 1) revealed that the best treatment for growing wheat is to application of zinc to the soil and as foliar spray (T3). Several important factors, like grain yield, thousand grain weight, plant height, and flag leaf area, were higher in Zn-treated wheat genotypes. Both Akbar-19 (plant height = 105 cm, grain yield = 26.5 g, flag leaf area = 52 cm², tillers = 13) and SD-75 (plant height = 108 cm, grain yield = 28.1 g, flag leaf area = 54 cm², tillers = 14) were highly responsive to Zn under T3, suggesting that they were promising varieties for making food more nutrient-richer. Only Zn foliar treatment also show that it has a significant effect on yield. However, Ghazi-19 and Bakhar-star exhibit poorly, suggesting they have less ability to uptake Zn.

Comparison of Zinc fertilizer rate

Flag leaf size and peduncle length were larger with Zn application from T3; possibly due to boosted photosynthesis activity and the process of filling the grains. No changes were observed in plant height. The finding revealed that different genotypes behave differently to the provided treatment. Both Fakhar-e-Bakhar and SD-166 obtained moderate increases in Zn, making those two less perfect than Akbar-19 and SD-75. Likewise, a highly significant correlations was observed among traits related to yield. The height of the plant was found to positively correlate with peduncle length, the number of spikelets per spike, thousand grain weight, and also with the number of tillers, the mother spike weight and grain zinc content. However, the length of the peduncle and flag leaf area had negative correlation with mother spike weight.

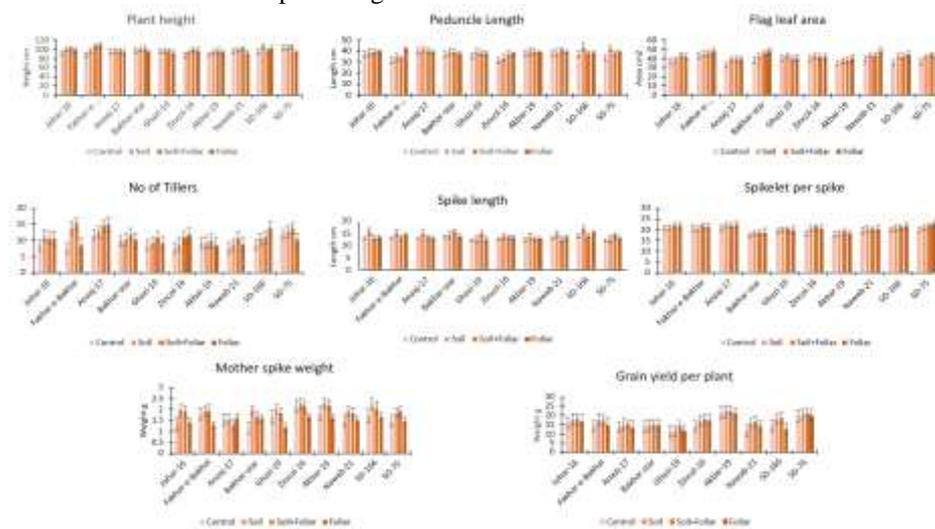


Figure1. Graphical representation of the Mean performance of genotypes for all the traits under applied treatments

Furthermore, tillers in a plant were not significantly linked to mother spike weight. There was a strong positive correlation between the length of the spike and the weight of the mother spike, but spike length was not significantly linked to thousand-grain weight and grain yield. Additionally, there was a very strong positive association between the weight of a thousand grains and grain yield. Grain zinc content showed a significantly positive correlation with all the traits except flag leaf area, suggesting a positive effect on yield and biofortification efforts.

The scree plot (Figure 3) showed the eigenvalues and cumulative variance explained by all the principal components obtained from the dataset. The first principal component (PC1) took the largest proportion of the total variance, 25.5 percent, followed by the second (PC2) with 17.9 percent, and the third (PC3) with 15.4 percent. Together, the first three components described a total of 58.8 percent of the variance. All the other components contributed increasingly less to the overall variance, with none of them explaining more than 12 percent.

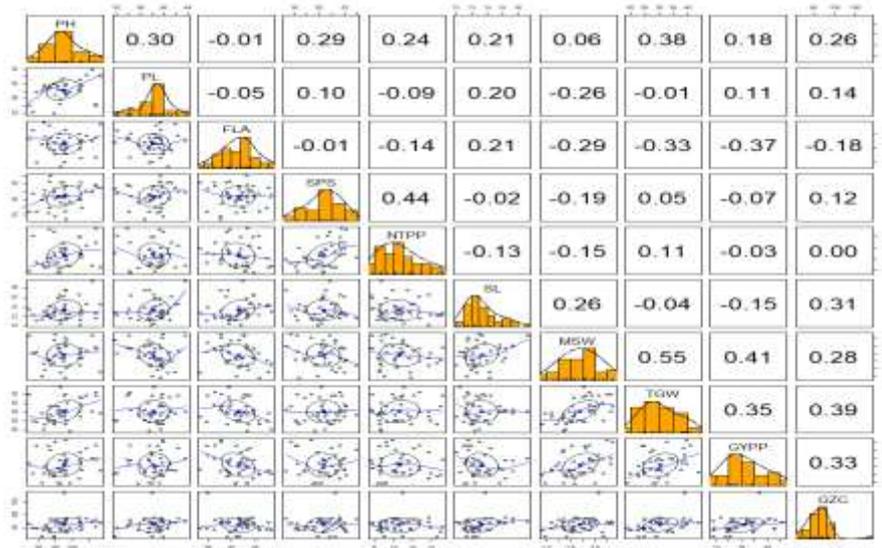


Figure 2. Correlation of all the traits PH (plant height), PL (peduncle length), FLA (flag leaf area), SPS (spikelets per spike), NTPP (no. of tillers per plant), SL (Spike Length), MSW (mother spike weight), TGW (thousand grain weight), GYPP (grain yield per plant), GZC (grain Zn content).

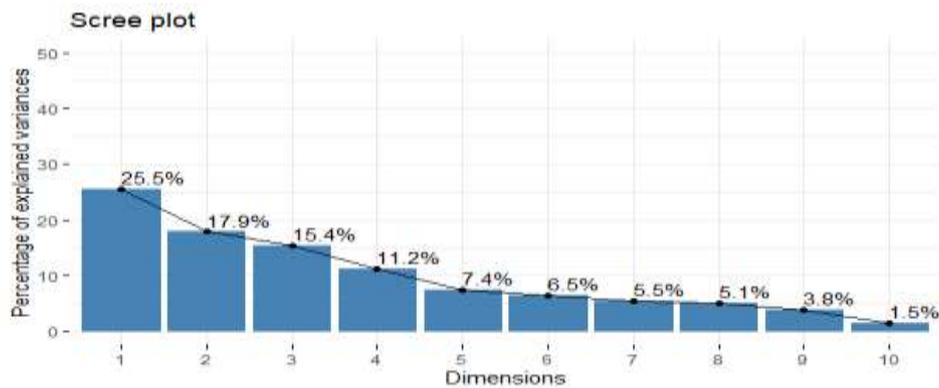


Figure 3. Scree plot for Percentage of explained variances by each principal component.

Moreover, the PCA variable (Figure 4) plot demonstrates that PC1 (25.5%) was determined mainly by yield-related traits, namely, TGW, MSW, and GYPP, that was strongly correlated with each other and subsequently contribute to a primary direction of variability within the data. The secondary traits like SPS, PH, NTPP and GZC stand out separately in PC2 (17.9%) adding less dominant variation. The intensity of the color (cos2) revealed that TGW and MSW were the most representative and reliable elements prescribed by these two components. Further, PCA biplot (Figure 5) indicates there was a correlation between TGW, PH, MSW, GYPP on the right (rightward variables), whereas the group C and F associate with upward/leftwards variables NTPP, FLA, SPS. This plot was a visual summary of the similarities and the differences among the group and which variables explain the differences.

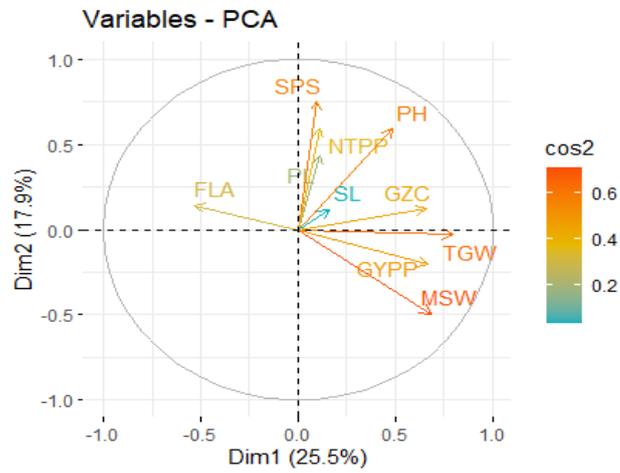


Figure 4. PCA biplot show contribution of key agronomic traits PH (plant height), PL (peduncle length), FLA (flag leaf area), SPS (spikelets per spike), NTPP (no. of tillers per plant), SL (spike length), MSW (mother spike weight), TGW (thousand grain weight), GYPP (grain yield per plant), GZC (grain Zn content)

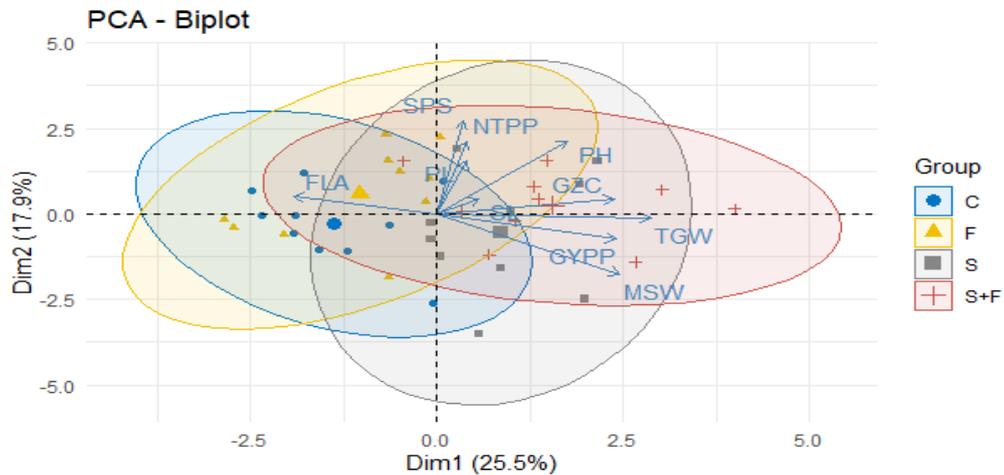


Figure 5. Principal Component Analysis (PCA) biplot showing trait associations and treatment groupings. The plot displays the first two principal components (PC1: 25.5% and PC2: 17.9% of total variance explained). Trait vectors indicate the direction and magnitude of variable contributions, while ellipses represent the 95% confidence regions of the four treatment groups: Control (C), Foliar (F), Soil (S), and Soil + Foliar (S+F). Trait clustering and group separation suggest potential treatment-specific responses and trait correlations: PH (plant height), PL (peduncle length), FLA (flag leaf area), SPS (spikelets per spike), NTPP (no. of tillers per plant), SL (spike length), MSW (mother spike weight), TGW (thousand grain weight), GYPP (grain yield per plant), GZC (grain Zn content)

Nutrient profiling of wheat grain for zinc content

Micronutrient profiling of wheat grain showed a significant positive response to soil and foliar application of zinc. Zn application caused a significant increase in grain zinc content and plant yield in wheat genotypes. Akbar-19 showed the highest zinc uptake in foliar and soil + foliar treatment of zinc, followed by SD-75, Zincol-16 and Nawab-21. Results shows that both synthetic derivatives SD-75 and SD-166 had a significant increase in zinc, while some local genotypes Johar-16, Fakhar-e-bakhar, Bakhar-star, and Ghazi-19 had poor zinc content. Moreover, foliar alone and soil + foliar application of zinc caused a significant increase in wheat genotypes, respectively, in comparison to soil application of zinc, which had no significant effect on grain zinc content.

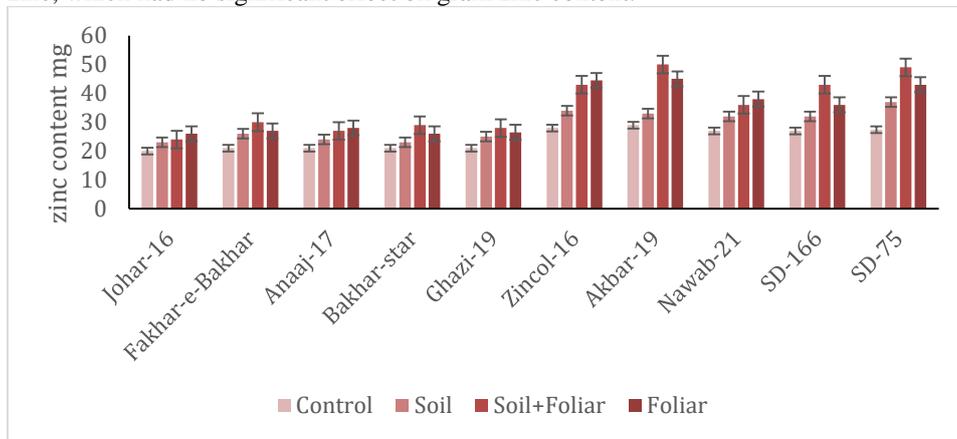
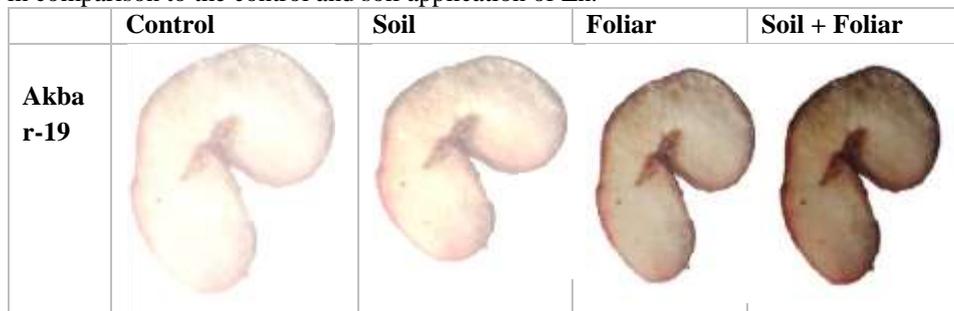


Figure 6. Graphical representation of Grain zinc content of genotypes at different treatment.

Microscopic Assessment

Zn localization (Figure 7) in wheat grain of four different wheat genotypes under applied Zn treatment in the form of ZnSO₄ showed that a higher content of Zn was found in the aleurone layer. Under applied Zn treatment, different localized Zn could also be seen in the wheat grain's aleurone layer and endosperm. When stained with diphenyl thiocarbazon solution, wheat grains exhibit a distinct red colour that is peculiar to Zn. The biofortified wheat genotype Akbar-19 and synthetic derivatives SD-75 had higher Zn. Results suggest that foliar and soil + foliar treatment had higher Zn content in wheat grain in comparison to the control and soil application of Zn.



Comparison of Zinc fertilizer rate

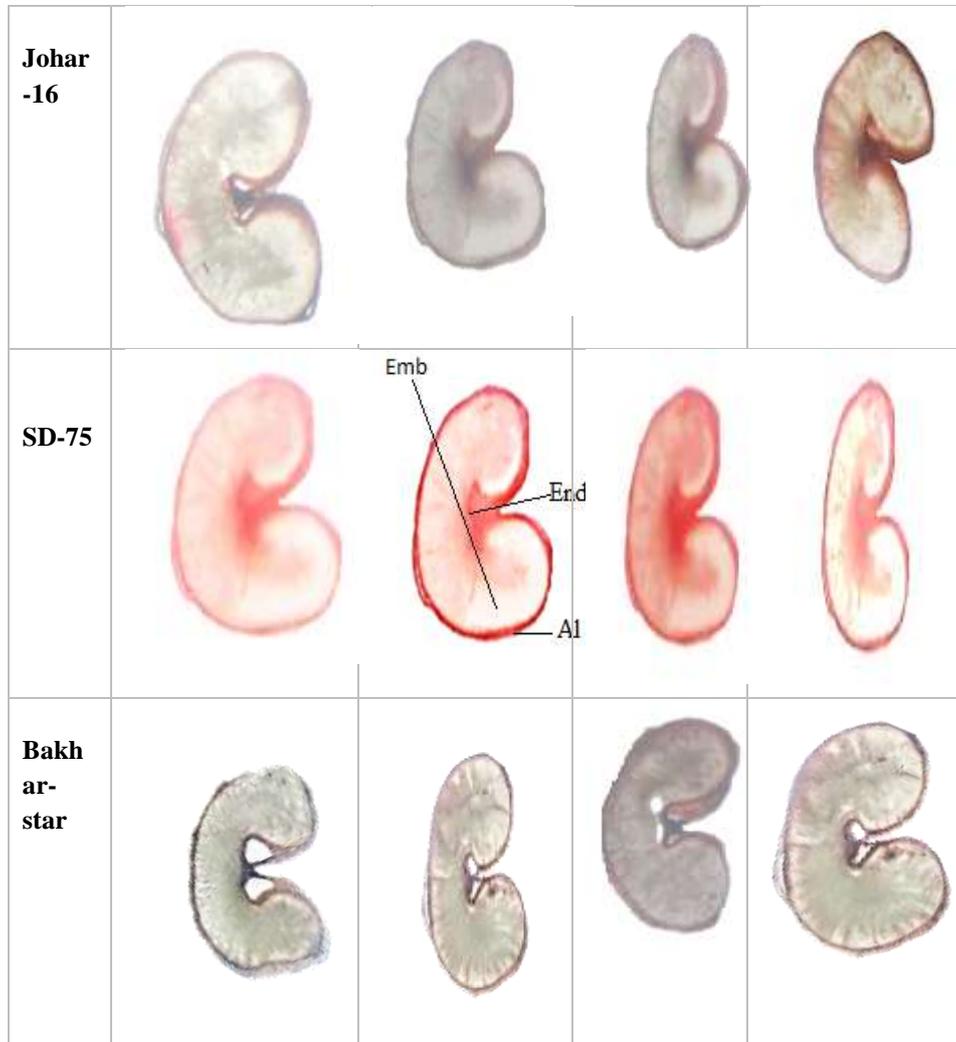


Figure 7. Localization of Zn in wheat grain under different treatments.

Discussion

The study highlights the significant impact of soil and foliar zinc application on enhancing physio-morphological traits and grain Zn concentration in wheat genotypes. Several studies demonstrate that integrating soil and foliar Zn application enhances key agronomic traits in wheat. For instance, it was reported that the combined application of Zn and potassium fertilizers significantly improved parameters like the number of grains per plant, spike length, thousand-grain weight, grain yield, and Zn uptake in wheat (Bashir et al., 2023). Similarly, it was found that foliar nano-Zn application combined with plant growth-promoting bacteria inoculation increased Zn concentration in shoots and grains, as well as improved grain yield and Zn use efficiency (Jalal et al., 2023; Upadhyay et al., 2024). The superior performance of the S+F treatment underscores its potential to address both root uptake limitations and direct foliar absorption, ensuring optimal nutrient availability. Among the ten wheat genotypes evaluated, Akbar-19 and SD-75 excelled under combined soil and foliar zinc application, achieving remarkable yield gains and elevated grain Zn content. In contrast, Ghazi-19 and Bakh-ar-star struggled across treatments, likely due to genetic or uptake limitations, (Hassan et al., 2019). Multi-year studies (Khoshgoftarmanesh et al., 2005) highlight cultivar diversity in yield, Zn

efficiency, and resilience critical for breeding semi-arid-adapted varieties (Fellahi et al., 2024). Stronger root systems correlated with higher yields (Noor et al., 2024), though grain Zn variability persists, underscoring the need for strategic genotype selection. Leveraging such genetic diversity through targeted breeding (Ivić et al., 2021) can optimize productivity while reducing fertilizer dependency.

Foliar zinc (Zn) application was found to be far more effective than soil methods, improving Zn concentration by 12–72% (up to 64 mg kg⁻¹), as demonstrated by Kong et al. (2024). This approach bypasses soil constraints like Zn fixation in calcareous soils by directly delivering nutrients (Ozturk et al., 2006; Yang et al., 2011). While soil Zn improves structural traits (e.g., tiller count, spike weight), it has a minimal impact on grain Zn, necessitating integrated strategies. Recent research emphasizes soil-specific considerations, as Zhang et al. (2024) found that diammonium phosphate (DAP) in calcareous soils enhances post-anthesis Zn uptake and bioavailability. However, cultivar variability in Zn absorption (0.7–1.1%) and grain translocation (23–66%) affects outcomes (Kamran et al., 2023). Combined foliar-soil approaches, soil type and phosphorus interactions, could maximize biofortification efficacy while addressing global Zn deficiency (Sánchez-Palacios et al., 2023; Singh et al., 2023).

Correlation and principal component analysis highlight that grain yield in wheat was strongly linked to traits such as mother spike weight, thousand grain weight, and tiller number, making these reliable indicators for selecting high-yielding genotypes. Multivariate statistical tools like PCA, especially under zinc-enriched conditions, had proven effective for clustering superior genotypes and identifying key trait associations, supporting more targeted and efficient breeding strategies for developing resilient, high-yielding, and nutritionally improved wheat varieties (Tadesse et al., 2023). Micronutrient profiling and localization studies demonstrated that Zn accumulates predominantly in the aleurone layer of wheat grains, with higher concentrations observed under foliar and S+F treatments. This is consistent with previous studies, which reported similar Zn localization patterns in wheat grains (Kiran et al., 2021). The enhanced Zn content in biofortified genotypes like Akbar-19 and SD-75 suggests their potential to address dietary Zn deficiencies, particularly in populations reliant on cereal-based diets.

Conclusion

It has been found that application of zinc in different forms, especially when a combination of soil and foliar spray depicts strong boosts in the growth and yields of wheat, as it increases plant height, flag leaf size, weight of the mother spike, thousand-grain weight, and grain yield per plant. Since Akbar-19 and SD-75 responded most to Zn application, they are good options for biofortification programs. Using foliar application was the best way to increase Zn in grain, leading to significant accumulation in the aleurone layer and endosperm. Evidence indicates that using zinc in an integrated way can heighten wheat yield and improve nutrient content in Zn-deficient soil so that micronutrient malnutrition can be addressed.

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Competing Interests

The authors declare that the research was conducted without any commercial or financial relationships that could be perceived as a potential conflict of interest.

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Consent to participate: All authors participated in this research study.

Consent for publication: All authors submitted consent to publish this research.

Data availability statement: The data presented in this study are available on request.

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