

## Zinc biofortification in upland rice: from paddy rice to white rice

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**Abbreviations:** AE-agronomic efficiency; APE-agro-physiological efficiency; COV<sub>xy</sub>-phenotypic covariance; CV-coefficient of variation; GY-grain yield; PH-plant height; rgg-accuracy; r<sub>xy</sub>-phenotypic correlations; TGW-1000 grain weight; ZnA-accumulation of Zn in grains; ZnAE-Zn absorption efficiency by the grain.

### Abstract

Hidden hunger affects approximately two billion people worldwide, with zinc (Zn) deficiency being one of the most prevalent mineral deficiencies, impacting around 1.2 billion individuals and leading to severe health disorders. Despite its widespread consumption and nutritional importance, rice (*Oryza sativa* L.) naturally contains low levels of Zn, limiting its contribution to human dietary needs. This study aimed to assess the genetic variability and Zn absorption potential in elite upland rice lines after agronomic biofortification, under different processing conditions. Five lines from the upland rice breeding program were evaluated in two environments, contrasting in terms of zinc content in the soil (2,70 and 1,20 mg dm<sup>-3</sup>), using a 5 × 2 factorial scheme in a randomized block design with three replications. The traits assessed included plant height, 1000 grain weight, grain yield, germination, and Zn content in paddy, brown, and white rice. Genetic variability in Zn accumulation was observed, with the ERF 85-15 line reaching 47.39 mg kg<sup>-1</sup> in brown rice and 35.25 mg kg<sup>-1</sup> in white rice. The estimated contribution of ERF 85-15 to daily Zn intake, based on a global per capita rice consumption of 147,7g day<sup>-1</sup> was 63.6% for brown and 47.4% for white rice. These findings highlight the potential of genetic and agronomic biofortification as sustainable strategies to combat Zn deficiency without requiring changes in consumer habits.

### Introduction

Zinc (Zn) is an essential micronutrient for both plants and humans. In plants, it plays a critical role in enzyme activation, carbohydrate metabolism, protein synthesis, auxin production, pollen formation, and stress tolerance regulation (Tsonev and Cebola Lidon, 2012). In humans, Zn deficiency is one of the most widespread nutritional disorders, affecting approximately 1.2 billion people worldwide. This deficiency can lead to severe health issues, including stunted growth, cognitive impairments, weakened immune system, and increased susceptibility to infections such as pneumonia (Wairich et al., 2022; Wani et al., 2017).

Rice (*Oryza sativa* L.) is a staple food for over half of the global population, providing a significant portion of daily caloric intake, particularly in at least 33 countries where it accounts for up to 80% of calories consumed (Lidon et al., 2018). Rice is not only a vital source of energy but also a key component in the fight against hunger and malnutrition.

Despite its widespread consumption, rice often lacks sufficient levels of essential micronutrients, including Zn, to meet human dietary requirements (Cakmak and Kutman, 2018). The nutritional gap is worsened by the natural Zn deficiency in many agricultural soils (Félix et al., 2023). Biofortification, through genetic improvement and

agronomic practices, offers a promising solution by increasing Zn content in rice grains, helping to reduce global micronutrient deficiencies (Bhardwaj et al., 2022).

Agronomic and genetic biofortification are complementary strategies to enhance Zn content in crops, focusing on its absorption, translocation, and accumulation in grains (Cakmak et al., 2018\*; Jalal et al., 2020). Their success relies on selecting genotypes highly responsive to these approaches for efficient nutrient uptake and storage.

This study evaluates genetic variability and Zn absorption in elite upland rice lines by assessing Zn content from paddy to white rice. The goal is to identify high-Zn genotypes to support genetic biofortification efforts and enhance global nutritional security.

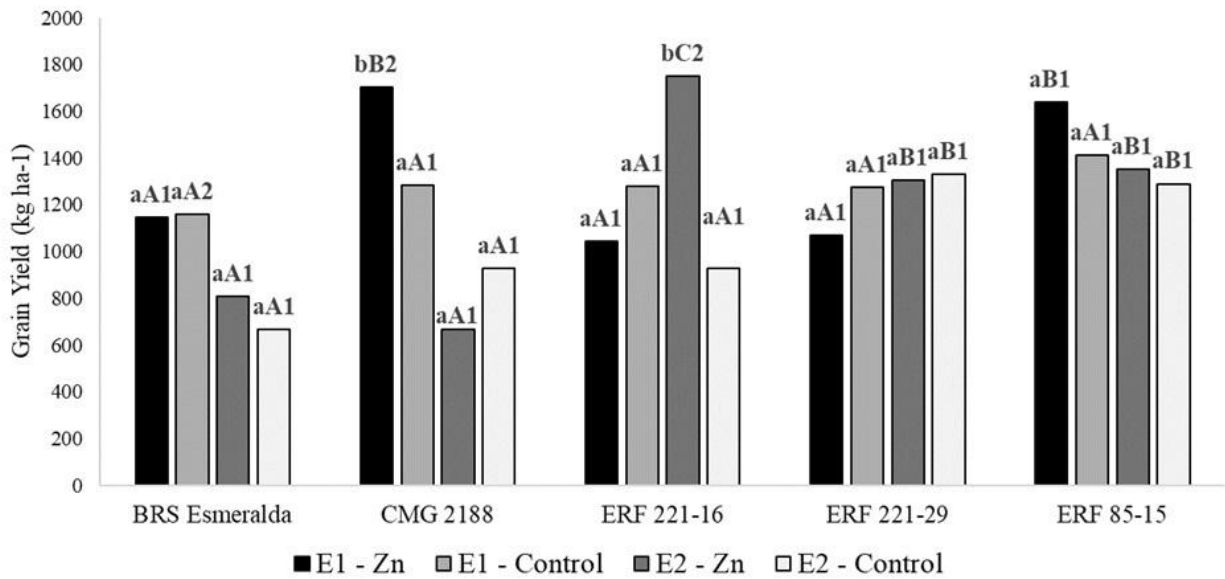
### Results

In this study, all traits showed CVs below 20% (Supplementary Table S3) (Pimentel-Gomes, 2009). Accuracies above 0.92 were obtained, indicating high reliability of selection (Resende & Alves, 2022).

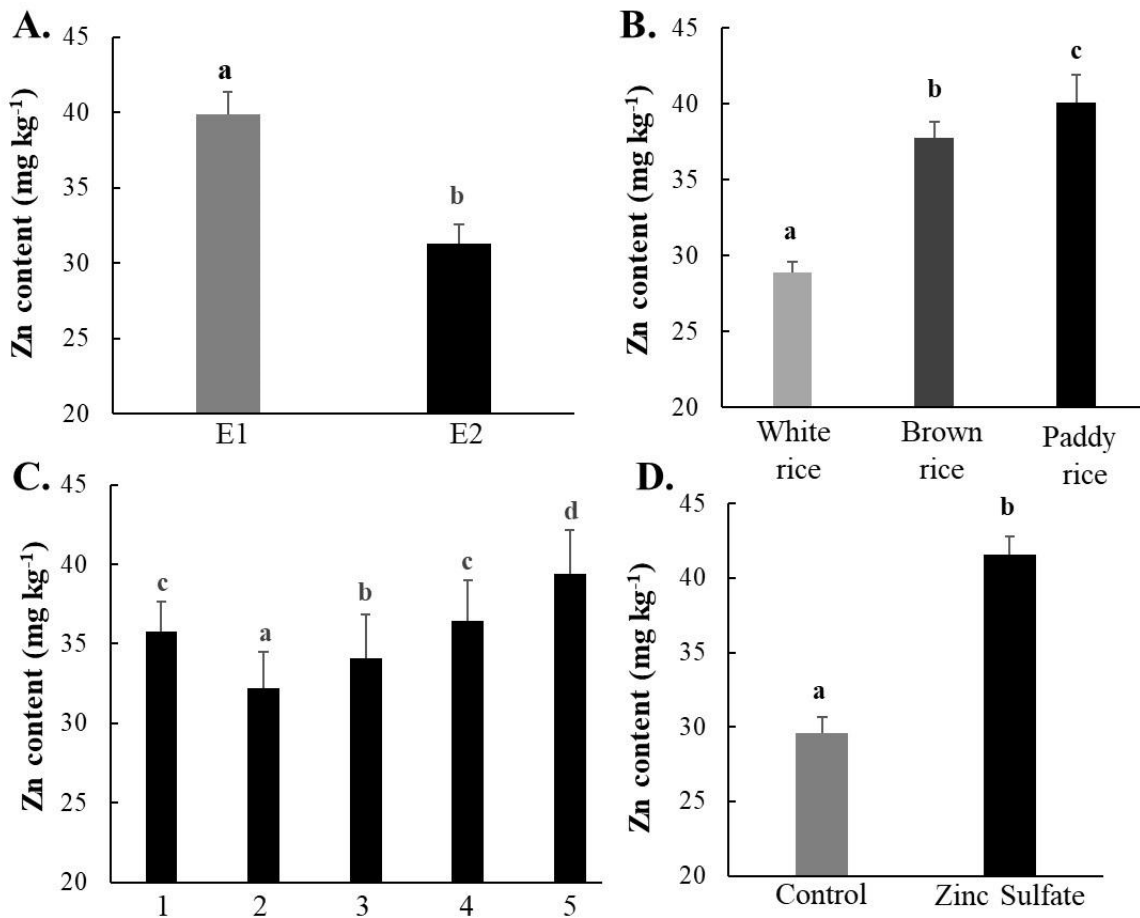
Significant genotypic variance ( $p \leq 0.01$ ) for all agronomic traits confirmed genetic variability among the lines. A significant genotype-by-environment interaction ( $p \leq 0.01$ ) for GY indicated variation in line performance across environments.

While there was no significant difference ( $p > 0.01$ ) observed in any agronomic traits with the application of Zn sulfate, the triple interaction of genotypes, environments,

and Zn sulfate application proved to be significant for GY (Fig. 1).



**Figure 1.** Grain yield of upland rice lines evaluated in two environments (E1 and E2), with (Zn) or without (Control) Zn application. The means depend on the breakdown of genotypes x environments x Zn applications interaction. Means followed by different lowercase letters in the same environment, means followed by different capital letters in the same Zn application factor and means followed by different numbers in the same genotype indicate significant differences ( $p < 0.05$ ) by the Skott-Knott test.



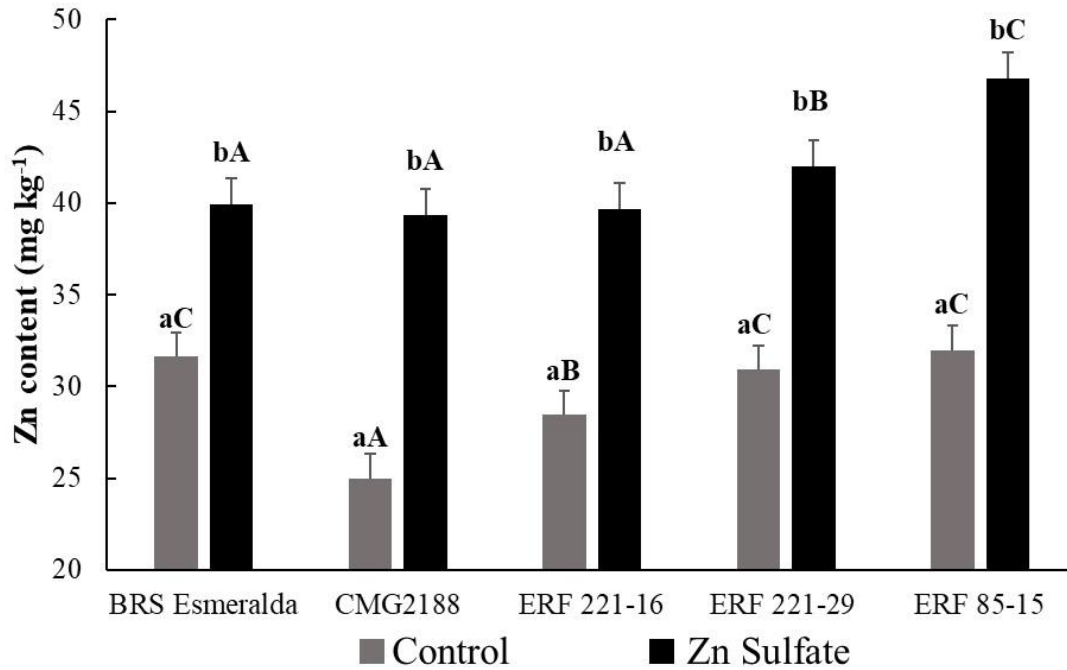
**Figure 2.** Zn content obtained from upland rice lines in relation to sources of variation (A) environments, (B) rice processing methods, (C) genotypes, being the lines BRS Esmeralda (1), CMG 2188 (2), ERF 221-16 (3), ERF 221-19 (4) and ERF 85-15 (5), and Zn application (D). Different letters indicate significant differences ( $p < 0.05$ ) between genotypes by the Skott-Knott test. The error bars indicate  $\pm$  Standard Error.

Analysis of Zn content in rice grains revealed significant effects ( $p \leq 0.01$ ) from genotypes, environments, Zn application, and processing methods (Supplementary Table S4). The coefficient of variation was 9.18%, with an accuracy of 0.98.

Zn content in grains was 21.5% higher in E1 than in E2 (Fig. 2A), and decreased by 5.9% after husking and 27.9% after polishing (Fig. 2B). Line ERF 85-15 showed the highest Zn accumulation (Fig. 2D), while foliar Zn sulfate application

increased Zn content by 28.79% compared to the control (Fig. 2C).

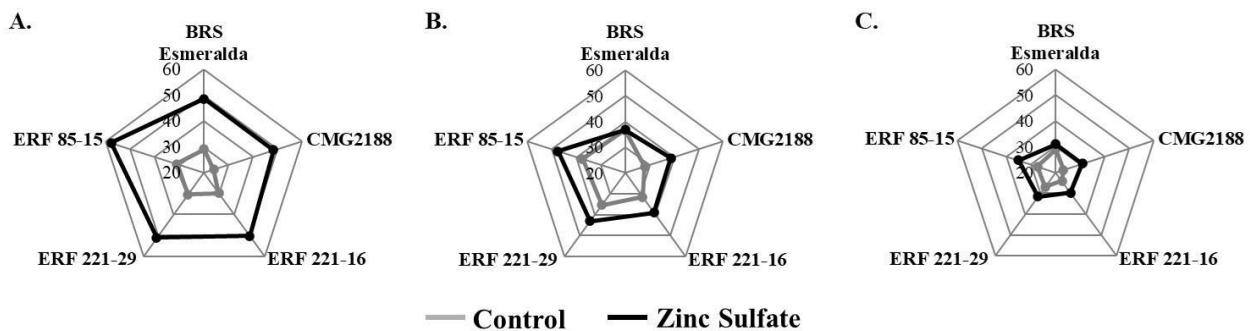
The genotype  $\times$  Zn application interaction showed complexity, with Zn sulfate significantly increasing grain Zn content in all lines by 20.87% to 36.49% (Fig. 3). ERF 85-15 and ERF 221-29 had the highest Zn levels under foliar fertilization, averaging 46.82 and 42.01  $\text{mg kg}^{-1}$ , respectively.



**Figure 3.** Zn content related to the breakdown of genotypes  $\times$  Zn applications interaction obtained in the grains of upland rice lines. Different lowercase letters between Zn applications and different uppercase letters between genotypes indicate significant differences ( $p < 0.05$ ) by the Skott-Knott test. The error bars indicate  $\pm$  Standard Error.

Figure 4 (A-C) shows Zn content means by genotype under Zn sulfate application and control across rice processing stages. Zn sulfate significantly increased Zn levels in paddy rice for all genotypes (Fig. 4A). In brown and white grains, increases ranged from 22.29% to 38.54% and 17.48% to

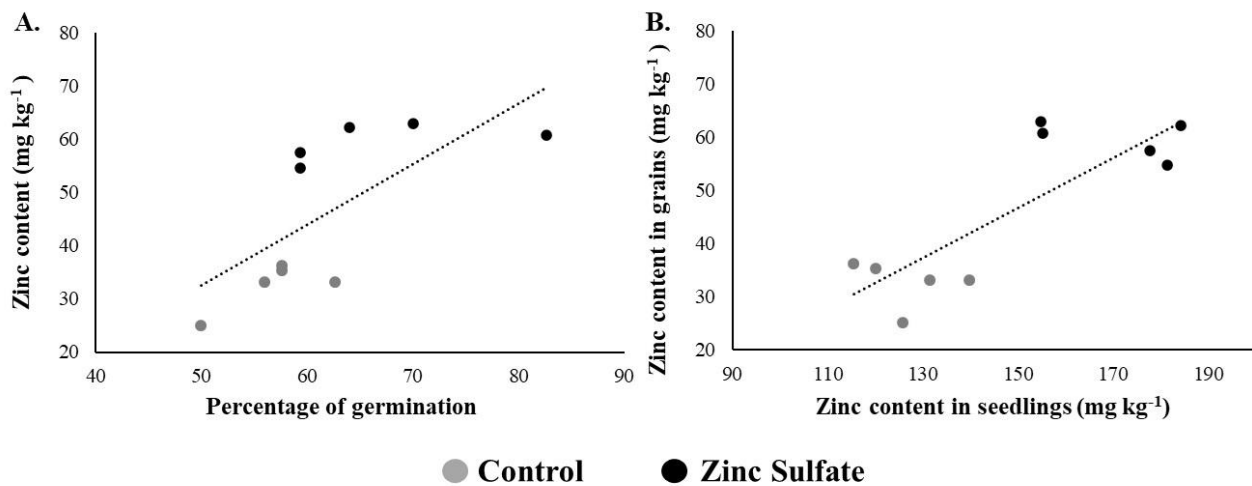
34.23%, respectively, except for BRS Esmeralda, which showed no significant change. CMG2188 responded most to Zn application, while ERF 85-15 maintained the highest Zn content overall.



**Figure 4.** Zn content in rice grains from different grains processing, paddy rice (A), brown rice (B) and white rice (C), from plants with foliar application of Zn sulfate and without application (control).

This study analyzed Zn content in seedlings derived from seeds of both biofortified and non-biofortified plants. Germination percentage was significantly affected ( $p \leq 0.01$ ) by genotype, Zn sulfate application, and their interaction, as shown in Supplementary Table S5.

Furthermore, the Zn content in the grain exhibited a significant positive correlation with the percentage of germination ( $r=0.69$ ) and the Zn content in the seedlings ( $r=0.82$ ) (Fig. 5).



**Figure 5.** Correlation between Zn content in grains and percentage of germination (A) and between Zn content in grains and Zn content in 10 days seedlings (B).

Regarding AE (Table 1), only ERF 221-29 showed negative efficiency in the joint analysis. A similar pattern was observed for APE. ZnAE ranged from 2.95% to 6.24%, with

ERF 85-15 (6.24%) and ERF 221-16 (5.26%) having the highest values.

**Table 1.** Effect of upland rice genotypes on Zn use efficiencies in two different environments, based on agronomic (AE, kg kg<sup>-1</sup>), agro-physiological (APE, kg g<sup>-1</sup>) and Zn absorption (ZnAE, %) efficiencies.

Lines	AE	APE	ZnAE
BRS Esmeralda	91.81	3.10	2.95
CMG 2188	114.27	2.62	4.35
ERF 221-16	410.39	7.80	5.26
ERF 221-29	-161.96	-5.45	2.97
ERF 85-15	205.29	3.29	6.24

## Discussion

Compared to other cereals, rice is a poor source of essential micronutrients, and it cannot meet the daily mineral intake needs demanded by humans (Cakmak and Kutman, 2018). Biofortification strategies to increase Zn content in grains include agronomic approaches, which rely on efficient fertilizer management, and genetic approaches, which involve selecting genotypes with higher Zn accumulation or greater absorption efficiency. The greater effectiveness of these different biofortification methods is reported when applied together (Calayugan et al., 2020; Félix et al., 2023). Thus, knowledge of genetic variability, as well as mineral concentration in the grain, is fundamental for plant breeding for the development of biofortified cultivars.

Genetic variability is essential for selecting superior genotypes, especially in the final stages of a rice breeding program, where lines from diverse populations are evaluated. This study revealed significant variability among genotypes for PH, TGW, and GY (Supplementary Table S3).

Although Zn sulfate application alone did not significantly affect the agronomic traits, grain yield (GY) was significantly influenced by the genotype × environment × Zn sulfate application interaction. Despite Zn's known roles in enhancing auxin metabolism, enzymatic activity (Umair Hassan et al., 2020•), and photosynthate translocation (Ali et al., 2021), this triple interaction limits conclusive interpretation of its isolated effect. Notably, ERF 221-16 showed a 46.9% increase in GY with Zn application in

environment E2, while no significant response was observed in E1 (Fig. 2).

A study comparing four Zn application methods across two environments and rice production systems reported a yield increase of approximately 30% compared to the control (Farooq et al., 2018). These findings support the hypothesis that Zn sulfate application can enhance upland rice productivity, highlighting the need for further research.

In Zn biofortification programs, grain yield (GY), grain quality, and high micronutrient content are key selection traits. In brown rice, approximately 57% of Zn is in the endosperm, while the bran and embryo contain 34% and 9%, respectively. However, around 40% of total Zn is lost during processing, polishing, and whitening (Tripathy et al., 2020). As rice is a staple food for over half the global population, increasing Zn levels in white rice through biofortification offers a cost-effective and sustainable strategy to combat micronutrient malnutrition.

The Zn content in grains was influenced by the effect of the environment. The highest average levels of Zn content in rice grain are observed in E1, with an accumulation of 21.5% compared to E2 (Fig. 2A). In the analysis of the chemical and physical attributes of the soil, E2 presented a medium Zn content (<1.5 mg dm<sup>-3</sup>). Thus, under these conditions of inadequate Zn availability in the soil, the genotypes may not have been able to express their full potential for accumulating this micronutrient in the grain.

A high phenotypic correlation was evidenced between the Zn content before and after polishing, demonstrating that

the processing loss is relatively constant among genotypes and, consequently, estimates of Zn content in brown rice are effective indicators of the content in white rice. Similar trends have been reported in other studies, and considering Zn losses during polishing, genotypes with over 30 mg kg<sup>-1</sup> of Zn in brown rice are considered promising parents for biofortification breeding programs (Kumar et al., 2017; Maganti et al., 2020). In this study, all biofortified genotypes exceeded this threshold, with Zn content in brown rice ranging from 36.76 to 47.39 mg kg<sup>-1</sup> (Fig. 4).

Regarding Zn content in white rice, genotypes that present average concentrations greater than 25.1 mg kg<sup>-1</sup> are classified as high content (Maganti et al., 2020). In this study, when Zn sulfate was applied, all genotypes showed high Zn content in white grain (Fig. 4), ranging from 30.94 to 35.25 mg kg<sup>-1</sup>. The presence of genotypic variability for the trait is evident, with the possibility of success in the selection of upland rice lines for higher Zn content in grains. Genetic variability for Zn content has been explored in several crops with the aim of identifying the best genotypes with high micronutrient content. A variation of approximately 3.5 times in the Zn content in rice grains was identified in 939 genotypes, with Zn content varying between 15.9 and 58.4 mg kg<sup>-1</sup> (Graham et al., 1999). Variations of 6.3 to 24.4 mg kg<sup>-1</sup> and 15.3 to 58.4 mg kg<sup>-1</sup>, in iron and Zn content respectively, were reported in a study carried out with 192 brown rice genotypes (Nachimuthu et al., 2014).

The ERF 85-15 stands out because, in addition to presenting the highest Zn content in the grains at all stages of rice processing, the plants also showed high content (>25.1 mg kg<sup>-1</sup>). The prevalence of Zn deficiency in humans is related to low intake of nutrients. It is estimated that Zn deficiency contributes to approximately half a million deaths per year among children under 5 years of age (Krebs et al., 2014). The Recommended Dietary Allowance (RDA) of Zn is approximately 11 mg day<sup>-1</sup> for adults, however, this requirement can be up to three times higher depending on the bioavailability of Zn in the food (Cakmak and Kutman, 2018). When biofortified, ERF 85-15, can supply 63.6% and 47.4% of the daily Zn intake with brown and white rice, respectively, based on a global per capita rice consumption of 147.7 g day<sup>-1</sup> (FAO, 2023). Considering the average per capita rice consumption in Brazil of 131.4 g day<sup>-1</sup> (CONAB, 2021), the same biofortified line would supply 92.6% of the daily requirement for the nutrient for children in this age group, considering the RDA of 5 mg day<sup>-1</sup> of Zn for children of 4 to 8 years of age (Padovani et al., 2006).

There is limited research on the effects of biofortification with Zn on seed quality, particularly in upland rice. In cereals, Zn is rapidly mobilized to the roots and coleoptile during germination. This increased demand for Zn can be attributed to highly active cell division and elongation, as well as high protein synthesis (Imran et al., 2017). In *Triticum durum*, Candan et al. (2018) observed reduced growth in seedlings from treatments with low Zn content seeds. Furthermore, the results of this same study clearly indicate that high Zn reserves in seeds may be necessary to minimize abiotic/biotic stress effects during germination. Similarly, Ei et al. (2020) found that selenium combined with Zn provided a significantly positive effect on the germination percentage of biofortified rice seeds. In this study, it was observed that the Zn content in the grain had a significant positive correlation with the percentage of germination ( $r=0.69$ ) and with the Zn content in the

seedling after germination ( $r=0.82$ ) (Fig. 5). These results suggest that biofortification, in addition to producing more nutritious grains, can play a beneficial role in relation to seed quality.

To study Zn use efficiency, three efficiencies were calculated as proposed by Fageria (2009), agronomic (AE), agro-physiological (APE) and Zn absorption (ZnAE). The ERF 221-16 presented higher values for both AE and AFE. These results reflect what was previously discussed regarding the increase in grain yield when Zn sulfate was applied to this genotype, especially in the E2 environment. For ZnAE, ERF 85-15 showed greater efficiency compared to the other lines. Studies on Zn use efficiency are scarce. However, for rice, it is possible to find values in which ZnAE varied from 0.73 to 1.33% (Farooq et al., 2018) and 6.40 to 9.10% (Zulfiqar et al., 2021), when just one foliar application of 0.5% Zn was considered. Furthermore, Fageria (2009) reports that the absorption efficiency for micronutrients is relatively low (5 to 10%) compared to that for macronutrients (10 to 50%) due to several factors.

It is important that biofortification strategies consider the potential antagonistic relationship between nutrients. The Zn content in the grain has an antagonistic influence with Fe content, that is, when the Zn content increases, the Fe content tends to decrease (Jalal et al., 2020; Ramzan et al., 2020). However, in the present work, the existence of a significant negative correlation between the content of Fe and Zn in the grain was not observed. Nevertheless, additional studies must be conducted when considering the simultaneous application of the two elements aiming at the biofortification of both nutrients.

## Material and methods

### Experimental locations

The experiments were conducted in two environments: Lavras (E1), at 918 m altitude (21°14'S, 45°00'W), and Lambari (E2), at 887 m altitude (21°58'S, 45°21'W), both located in Minas Gerais state, Brazil. The chemical and physical soil attributes evaluated at the 0–20 cm depth at both locations following the methodologies of Teixeira et al. (2017) are presented in Supplementary Table S1. Based on the results, fertilization was applied according to crop and regional recommendations (Supplementary Table S2).

### Genotypes

Five elite lines, BRS Esmeralda, CMG 2188, ERF 221-16, ERF 221-19, and ERF 85-15, were selected from the Value of Cultivation and Use (VCU) trials of the Upland Rice Breeding Program (MelhorArroz). Their selection was based on previous findings by Félix et al. (2023), which highlighted their efficiency in micronutrient uptake and translocation to the grains.

### Experimental Design and Management

The experiment followed a randomized complete block design with three replications in a 5 × 2 × 2 factorial scheme, comprising five lines with and without Zn sulfate (ZnSO<sub>4</sub>·7H<sub>2</sub>O) application, evaluated in two environments. Each plot had five 4.0 m rows spaced 0.4 m apart, with the outer rows discarded at harvest to avoid varietal mixing, resulting in a usable area of 4.8 m<sup>2</sup>. Sowing density was 80 seeds/m, and plots were spaced 0.8 m apart to reduce drift during foliar applications. Crop management followed local commercial practices, including sprinkler irrigation as needed. Two foliar applications of Zn sulfate (ZnSO<sub>4</sub>·7H<sub>2</sub>O) were administered at a dosage of 3 kg ha<sup>-1</sup>, at the R3 and R7

stages, corresponding to panicle elongation and the grain filling, respectively, aimed to enhance micronutrient accumulation in the grains.

The Zn sulfate solution was prepared in the laboratory to achieve a concentration of 1 g L<sup>-1</sup> of Zn in the mixture. Additionally, 5 mL of Assist® adjuvant was added per liter of solution.

### Traits Evaluated

Plant height (PH, cm), measured as the average of five randomly selected plants from soil to the tip of the tallest panicle.

1000 grain weight (TGW, g), estimated from ten random samples of 100 grains each; and grain yield (GY, kg ha<sup>-1</sup>), calculated at 13% moisture and extrapolated to 10,000 m<sup>2</sup>.

Germination percentage: Evaluated using three replications of 50 seeds placed in germitest paper rolls moistened with distilled water (2.5× paper weight) and incubated in a digital germinator at 25 °C. Normal seedlings were counted on the 5th and 10th days. Following the germination test, the seedlings underwent a drying process in an oven set at 60°C to analyze Zn content.

Zn content: determined in rice grains and seedlings using the USEPA 3051A method (USEPA, 2007). From each plot, 30 g of grain yielded 15 g of brown and 15 g of white rice. For digestion, 0.5 g of ground sample was mixed with 5 mL of HNO<sub>3</sub> (≥65%) in PTFE tubes and digested via microwave (CEM Mars-5) at 0.76 MPa for 15 min. After cooling, 5 mL of double-distilled water was added. Zn was quantified by ICP-OES (Spectro Blue, Germany), with accuracy verified using NIST SRM 1573a. The same extracts were also used to measure Ca, Mg, P, S, K, Cu, Fe, and Mn.

Zn use efficiency: the accumulation of Zn in grains (ZnA) (mg ha<sup>-1</sup>) was calculated by multiplying the Zn content in grains (mg kg<sup>-1</sup>) by the GY (kg ha<sup>-1</sup>) (Ducloux et al. 2016). Additionally, agronomic efficiency (AE, kg kg<sup>-1</sup>), defined as the GY obtained per kg of nutrient applied, agro-physiological efficiency (APE, kg g<sup>-1</sup>) defined as the GY obtained per g of nutrient absorbed, and Zn absorption efficiency by the grain (ZnAE%), defined as the percentage of nutrient absorbed from the nutrient applied, were calculated, according to Fageria (2009), using the following equations:  $AE = \frac{GY_{Zn} - GY_{0}}{N_{applied}}$ ,  $APE = \frac{GY_{Zn}}{N_{absorbed}}$ , and  $ZnAE\% = \frac{N_{absorbed}}{N_{applied}} \times 100$ . Where  $GY_{Zn}$  and  $GY_{0}$  represent the grain yield with and without Zn application, respectively;  $N_{applied}$  is the applied dose of Zn; and  $N_{absorbed}$  are the absorbed Zn with and without Zn application, respectively.

### Statistical analysis

The data were tested for ANOVA assumptions, including normality, independence, and homogeneity of residual variances. Once these assumptions were met, analyses of variance were performed using R software (R Core Team, 2020) for agronomic traits (PH, GY, and TGW). In the statistical model, only the error was treated as a random effect, while genotype, block within environment, environment, and Zn application were considered fixed effects.

Additionally, an ANOVA was carried out to evaluate the Zn content in grains from different grain processes, according to the following statistical model:

Where  $y_{ijkl}$  is the value in the plot that received genotype  $i$ , in block  $j$ , within of the factor Zn application  $k$ , in location  $l$  and rice processing factor  $r$ ;  $\mu$ : constant associated with all observations;  $\alpha_i$ : genotype fixed effect ( $i = 1, 2, \dots, 5$ );  $\beta_j$ : effect of block  $j$ , in environment  $l$ ;  $\gamma_k$ : fixed effect of the Zn

application  $k$ ; ( $k = 1, 2$ );  $\delta_l$ : fixed effect of the environment  $l$  ( $l = 1, 2$ ); rice processing effect ( $= 1, 2, 3$ ); effect of interaction between genotypes  $i$  and environments  $l$ ;  $\epsilon_{ik}$ : effect of interaction between genotypes  $i$  and Zn applications  $k$ ;  $\zeta_{lr}$ : effect of interaction between environments  $l$  and Zn applications  $k$ ; effect of interaction between Zn applications  $k$  and rice processing;  $\eta_{ir}$ : effect of interaction between genotypes  $i$  and rice processing;  $\theta_{il}$ : effect of interaction between environments  $l$  and rice processing;  $\phi_{ikl}$ : effect of interaction between genotypes  $i$ , environments  $l$  and Zn applications  $k$ ; effect of interaction between Zn applications  $k$ , environments  $l$  and rice processing;  $\psi_{ikr}$ : effect of interaction between genotypes  $i$ , Zn applications  $k$ , environments  $l$  and rice processing error associated with the independent observation, that  $\sim N(0, \sigma^2)$ .

The precision of the experiment and genetic predictions were evaluated using coefficient of variation ( $Cv$ ) and accuracy ( $r$ ) (Resende and Alves, 2022), respectively. Estimates of phenotypic correlations ( $r_{xy}$ ) between traits were calculated using the expression  $r_{xy} = \frac{Cov_{xy}}{\sqrt{V_x V_y}}$ , where  $Cov_{xy}$  represents the phenotypic covariance between traits  $x$  and  $y$ , and  $V_x$  and  $V_y$  represent, respectively, the phenotypic variance of  $x$  and  $y$ .

### Conclusion

Foliar application of Zn sulfate proved effective in biofortifying upland rice grains. All genotypes showed increased Zn content in both brown and white rice, confirming the relevance of agronomic and genetic biofortification. ERF 85-15 exhibited the highest Zn uptake after application, leading to increased Zn concentrations in both grain types. As expected, rice polishing led to significant Zn loss, highlighting the superior nutritional value of brown rice. Additionally, the enhanced Zn content in seeds may improve germination and seedling vigor, suggesting that Zn biofortification not only enhances nutritional quality but also offers agronomic benefits.

### Declaration of Competing Interest

The authors declare that they have no competing interests related to the content of this manuscript. There are no financial or personal relationships that could inappropriately influence the research presented in this study.

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