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Agronomic Biofortification Increases Concentrations of Zinc and Storage Proteins in Cowpea Grains

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Abstract: Zinc (Zn) is crucial for both plant metabolism and human nutrition, with its deficiency being a global health concern. Strategies to increase its availability in food, such as agronomic biofortification, have gained prominence. This study evaluated the impact of foliar spraying of Zn [at full bloom stage: 0 (control) and 600 g ha^{-1}, as $ZnSO_4 \cdot 7H_2O$] on the nutritional quality of cowpea (*Vigna unguiculata* L. Walp.) grains. Field experiments involving 20 cowpea genotypes were carried out over two seasons in a Typic Quartzipsamment under a no-tillage system. The photosynthetic responses of cowpea plants and the concentrations of Zn, amino acids, sucrose, total sugars, and storage proteins (glutelin, albumin, prolamin, and globulin) in grains were analyzed. All genotypes showed enrichment of Zn in grains in response to $ZnSO_4 \cdot 7H_2O$ application compared to untreated plants. Foliar spraying of $ZnSO_4 \cdot 7H_2O$ during initial grain filling was ideal for increasing Zn concentration in grains and improving plant physiological processes. Additionally, Zn fertilization led to higher concentrations of storage and total amino acids and proteins in the grains, supporting the rational application of Zn in cowpea production to improve the nutritional quality of grains and increase plant productivity.

Keywords: *Vigna unguiculata*; total sugars; amino acids; grain quality; zinc

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1. Introduction

Zinc (Zn) is an essential element for the proper functioning of the human body, playing crucial roles in various physiological functions [1]. It acts as an enzymatic cofactor and is involved in around 10% of the body's proteins [2,3]. Its insufficiency correlates with various health issues, including stunted growth, impaired brain development, heightened vulnerability to infections, pregnancy complications, and reduced resilience against oxidative stress and aging [4–6]. Zinc scarcity, along with other micronutrient deficiencies, contributes to "hidden hunger", affecting billions of people worldwide, especially in developing countries [3].

Zinc is the most deficient plant micronutrient due to its low availability in the soils under natural conditions, with an average concentration of approximately 64 mg kg^{-1}, though this content may vary depending on the origin material of each soil [6,7]. This lack of Zn in the soil further aggravates its deficiency in the population, especially among those

with low income [8]. In regions where soil and crops suffer from Zn scarcity, mineral deficiency in humans is common [5]. According to the World Health Organization (WHO) [9], the recommended dietary intake of Zn is 11 mg per day for men and 8 per day for women. Pregnant and breastfeeding women need 12 mg per day. However, many people fail to meet these recommendations, and approximately one-third of the global population is at risk of Zn deficiency, which is more prevalent among children under 5 years old due to the increased demand for this mineral for growth and development. Each year, about half a million children in this age group die due to Zn deficiency [1].

Biofortification is an effective strategy to increase the content of micronutrients in edible plants [10–13]. Biofortification is typically pursued through three primary methods: traditional plant breeding, agronomic practices, and genetic engineering in plant breeding [14]. Agronomic biofortification stands out as one of the simplest and most economical methods to enhance micronutrient content in plants. This is achieved by directly supplying micronutrients to plants through the application of mineral or foliar fertilizers, or by enhancing the solubility and mobility of mineral elements in the soil [12]. However, it is important to note that the range of Zn concentration for plants is narrow, requiring careful selection of plant species as well as salt concentrations and forms of fertilizers to achieve crops with high nutritional quality. Additionally, in choosing the crop, it is also crucial to opt for those widely consumed by the population to ensure the success of biofortification programs [6,12].

Cowpea (*Vigna unguiculata* L. Walp.) is a crop that plays a fundamental role in the diet of millions of people, especially in arid and semi-arid areas of northern and northeastern Brazil, where soils are naturally poor in plant nutrients, like phosphorus, and magnesium. This legume has been the subject of studies in the area of biofortification due to its high nutritional value [15]. Furthermore, considering that Zn plays a crucial role in nitrogen (N) metabolism and protein synthesis, its application can positively influence the protein concentration in cowpea grains. Thus, Zn biofortification in this crop has the potential to increase the content of this mineral nutrient in grains, contributing to the improvement of nutritional quality and consequently human health [3].

In addition to the benefits for human health, Zn biofortification is also crucial for agricultural production. Zn plays a vital role in the growth and development of plants, influencing protein synthesis, hormone regulation, antioxidant metabolism, and the stability of cell membranes [1,6,13]. Plants deficient in Zn often exhibit reduced growth, lower yields, and increased susceptibility to diseases, negatively affecting agricultural production [5]. The application of Zn to deficient soils can improve plant vigor, resulting in higher yields [7]. Thus, ensuring adequate Zn levels for plants not only improves the nutritional quality of food but also makes agricultural production more sustainable.

There is a shortage of studies investigating the genotypic effects of foliar-applied Zn fertilization in cowpea genotypes and their potential to ameliorate Zn deficiency prevalent in certain populations. Exploring potential genotypic variations presents an opportunity to investigate Zn accumulation in grains and improve their quality, address hidden hunger, and strengthen human health. Moreover, improving the Zn content in cowpeas can increase crop productivity, as well-nourished plants tend to produce more. Field experiments aimed at enriching cowpea grains with zinc provide valuable insights both for biofortification programs and for strategies to increase agricultural productivity. Therefore, with this work, the objective was to evaluate the impact of Zn foliar sprays on the nutritional quality and yield of cowpeas in 20 genotypes, examining the physiological responses of the plant, as well as amino acids, sucrose, total sugars, and storage proteins (albumin, globulin, glutelin, and prolamins) in grains.

2. Materials and Methods

2.1. Experimental Area Description

The experiment was carried out over two consecutive years (2021 and 2022) at the Federal Institute of Mato Grosso do Sul in Nova Andradina, MS, Brazil (22°4′35″ S 53°27′33″ W). The soil in the experimental area was identified as a Typic Quartzipsamment [16]. To evaluate the soil's chemical properties, 30 subsamples were randomly collected from the top 20 cm layer of the experimental field. These subsamples were combined, thoroughly mixed, and analyzed according to the methodology of Raij et al. [17]. The soil texture analysis indicated 95 g kg^{-1} clay, 54 g kg^{-1} silt, and 810 g kg^{-1} sand. The chemical properties of the soil were measured as follows: the pH ($CaCl_2$ 0.01 M) was 4.8; phosphorus (resin), boron (hot water), copper (DTPA), iron (DTPA), manganese (DTPA), and zinc (DTPA) were 1.8, 0.5, 0.3, 16, 21, and 0.45 mg dm^{-3}, respectively; potassium (resin), calcium (resin), magnesium (resin), H + Al (SMP buffer), and cation exchange capacity were 0.5, 8, 3, 14, and 25.5 mmolc dm^{-3}, respectively. These measurements were performed following the procedures outlined by Raij [18].

2.2. Experimental Design and Treatments

The experiment setup was a randomized complete block design with three blocks, in a factorial scheme of 20 (twenty cowpea genotypes) × 2 (two doses of Zn), totaling 120 plots. Each plot consisted of five rows, each 4 m long, with a spacing of 0.45 m between rows. This study evaluated twenty cowpea genotypes with brown tegument grain color under two conditions: no zinc application (control) and foliar application of 600 g ha^{-1} Zn as zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$, Sigma-Aldrich, St. Louis, MO, USA). This concentration and source of Zn were chosen based on previous studies that demonstrated effectiveness in improving the nutrient content in bean plants [6]. Zinc via foliar spray was applied 43 days after sowing (DAS) in the first and 42 DAS in the second year (full bloom stage) using a CO_2-pressurized costal spray [15]. The required quantity of zinc (Zn) for each treatment, across all three replications, was measured and diluted in 8 L of water to prepare a stock solution. This stock solution was then divided equally into three portions of 2 L each. These solutions were subsequently applied to the planting furrows. For each plot, 400 mL of the solution was precisely distributed along each row of plants using a small, flexible polyethylene bottle fitted with a punctured cap [19]. The cowpea genotypes used in this research were cultivated and obtained from the EMBRAPA (Brazilian Agricultural Research Corporation, Brasília, Brazil) germplasm bank. Table S1 provides details regarding the provenance, maturation cycle, and breeding method of obtention for each genotype.

2.3. Cowpea Cultivation

Sowing took place in October 2022 (first year) and March 2023 (second year), with rows spaced 0.45 m apart and a density of 13 seeds per square meter under a no-tillage system. Fertilization at planting included 40 kg ha^{-1} of potassium as KCl, 50 kg ha^{-1} of phosphorus as a single superphosphate, and 60 kg ha^{-1} of nitrogen as urea, all applied in the planting furrow mechanically during sowing [15]. Emergence occurred 11 DAS. Leaf sampling was performed at 62 DAS in both years (at the full bloom stage) to analyze leaf gas exchange parameters and nutrient contents. The third trifoliate leaf, counted from the apex, was harvested, dried in an oven at 60 °C until it reached a constant mass, and then ground in a Wiley mill with a 1 mm sieve. Fifteen trifoliate leaves were randomly collected from 15 uniform plants per plot. Harvesting and plant height measurements were conducted at 70 days after sowing (DAS) in the first year and 72 DAS in the second year, corresponding to pod maturity. For harvesting, two homogeneous rows were selected from each plot, and all pods were manually collected; grains were then manually removed from the pods. The grains were dried in an oven at 60 °C to a constant mass and ground in a Wiley mill with a 1 mm sieve [20].

2.4. Gas Exchange Parameters

Gas exchange parameters were assessed utilizing an infrared gas analyzer (LI-6400XT, LICOR, Lincoln, NE, USA) during the time frame of 08:00 to 10:00 a.m. They were measured under a photon flux density (PPFD) of 1000 μmol m^{-2} s^{-1} and an air CO_2 concentration of 380 μmol mol^{-1}, all on the day of plant harvesting. Net photosynthesis rate (*A*), leaf stomatal conductance (g_s), and transpiration (*E*) were measured following the methodology outlined by Santos et al. [21].

2.5. Mineral Nutrient Analysis

Samples of dried and ground leaves and grains, each weighing 0.25 g, were precisely weighed and subjected to digestion in perfluoroalkoxy (PFA) liner material digestion tubes. Each tube contained 2 mL of 70% Trace Analysis Grade HNO_3, 1 mL of Milli-Q water, and 1 mL of H_2O_2. For the analysis of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), boron (B), copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn), the digested samples were diluted at a ratio of 1:10 using Milli-Q water. Data analysis was performed using Qtegra™ software version 7.1 (Thermo Fisher Scientific, Waltham, MA, USA), as described by Silva et al. [22].

2.6. Determination of Total Sugars and Sucrose in Grain

The quantification of sucrose was performed using the method outlined by van Handel [23]. In this method, 20 μL of the hydrophilic portion of the MCW extract, 500 μL of 30% KOH, and 2 mL of concentrated H_2SO_4 were added to a glass tube. The mixture was vortexed and then heated in an oven at 100 °C for 10 min. After cooling to room temperature, the absorbance was measured at 490 nm with a spectrophotometer (SP-220, Bioespectro™ brand, São Paulo, Brazil). The sucrose content was reported in mg g^{-1} DW.

Total sugar quantification followed the protocol described by DuBois et al. [24]. In this procedure, 20 μL of the hydrophilic portion of the MCW extract, 500 μL of 5% phenol, and 2 mL of concentrated H_2SO_4 were combined in a glass tube. The mixture was vortexed, and after cooling to room temperature, the absorbance was read at 490 nm using a spectrophotometer (SP-220, Bioespectro™). Results were expressed in mg g^{-1} DW. A standard sucrose curve was used to quantify both sugar and sucrose.

2.7. Determination of Total Amino Acids and Storage Proteins

The quantification of total free amino acids in the grains was based on the method by Yemm et al. [25]. In this process, 250 μL of the hydrophilic portion of the MCW extract, 500 μL of 0.2 M sodium citrate, 200 μL of 5% ninhydrin in ethylene glycol, and 1 mL of 0.0002 M KCN were mixed in a glass tube. The mixture was vortexed and then heated at 100 °C for 15 min. After cooling with tap water for 10 min, 1 mL of 60% ethanol was added, and the solution was mixed again by vortexing. The absorbance at 570 nm was measured using a spectrophotometer (SP-220, Bioespectro™). The concentration of amino acids in the grains was calculated using a methionine standard curve, with results expressed in mg g^{-1} DW.

For the extraction of storage proteins, 0.20 g of dried and ground grains were sequentially extracted with 12 mL of deionized water (for albumin determination), 5 mL of 5.0% NaCl (for globulin determination), 5 mL of 60% ethanol (for prolamin determination), and 3 mL of 0.4% NaOH (for glutelin determination). Protein concentration was determined using the Bradford method [26], with a BSA (bovine serum albumin) solution as the standard.

2.8. Statistical Analysis

The results underwent Anderson–Darling normality tests to assess their distribution, while Levene's test and variance analysis (F test) were employed to evaluate the homogeneity of variance. Treatment differences were compared using the Scott–Knott test at a significance level of 5%. These statistical analyses were conducted using R software v. 3.5.1 [27]. Graphical representations were generated using SigmaPlot 12.5 software (Systat Software, San Jose, CA, USA).

3. Results

The yield of cowpeas in response to foliar-applied Zn fertilization showed that all genotypes receiving $ZnSO_4 \cdot 7H_2O$ had significantly higher yields relative to those that did not receive the micronutrient foliar fertilization (Figure 1). Specifically, genotype 7, when subjected to $ZnSO_4 \cdot 7H_2O$ addition, recorded the highest yield in both crops, achieving a four-fold production increase compared to the treatment without $ZnSO_4 \cdot 7H_2O$ addition, with an average yield of 2150 kg ha^{-1} over the two crops. Genotype 8 exhibited the second-highest yield with $ZnSO_4 \cdot 7H_2O$ addition, being three times higher than the same genotype grown without $ZnSO_4 \cdot 7H_2O$ fertilization. Regarding genotypes that did not receive $ZnSO_4 \cdot 7H_2O$, the highest yields were observed in genotypes 11, 12, 15, 16, 17, 19, and 20 in the 2022 crop (Figure 1a), and in genotypes 12, 16, 17, 18, 19 and 20 in the 2023 crop (Figure 1b). These same genotypes, when subjected to $ZnSO_4 \cdot 7H_2O$ addition, showed the lowest yield relative to other genotypes that also received $ZnSO_4 \cdot 7H_2O$. It is important to emphasize that foliar-applied $ZnSO_4 \cdot 7H_2O$ resulted in higher yields in all evaluated genotypes.

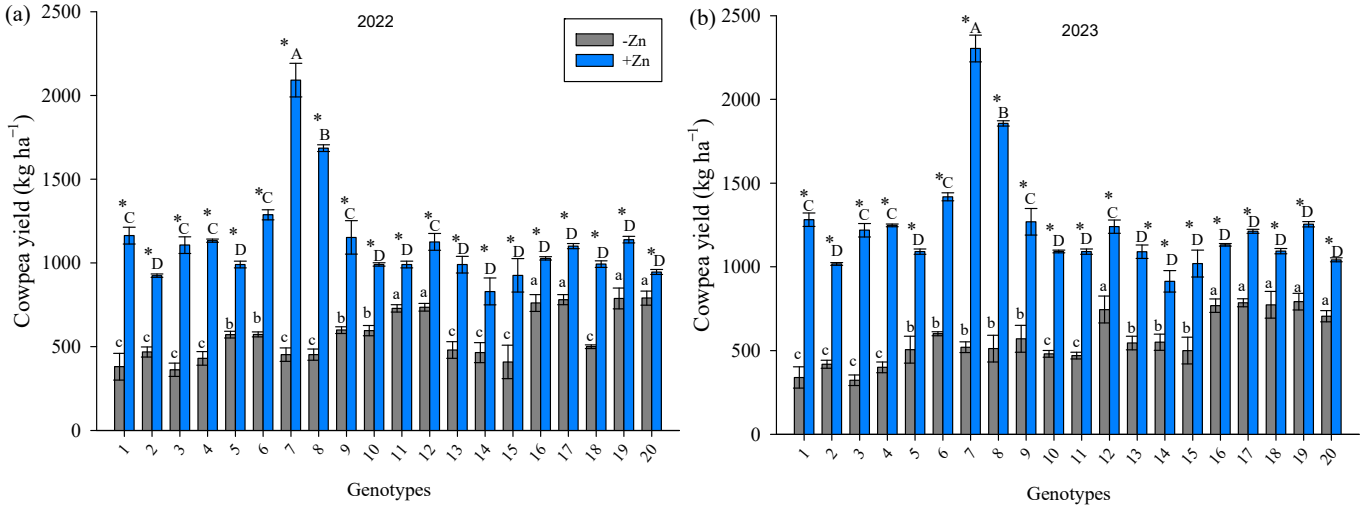

Figure 1. Cowpea yield during the first (**a**) and second (**b**) growing seasons for twenty cowpea genotypes in response to $ZnSO_4 \cdot 7H_2O$ application. Error bars represent the standard error of the mean (n = 3). Different letters denote significant differences between means as determined by the Scott–Knott test ($p \leq 0.05$). Uppercase letters compare genotypes with Zn application, while lowercase letters compare genotypes without Zn application. * indicates a significant difference between means of the same genotype with and without Zn application according to the Scott–Knott test ($p \leq 0.05$).

Zinc concentration in the leaves and grains of cowpeas was higher in genotypes subjected to $ZnSO_4 \cdot 7H_2O$ addition, highlighting the success of biofortification (Figure 2). In both crops, genotype 7 treated with $ZnSO_4 \cdot 7H_2O$ showed the highest Zn concentration in the leaves, followed by genotypes 3, 10, 15, and 19. These genotypes exhibited a higher Zn concentration in the leaves compared to those that did not receive $ZnSO_4 \cdot 7H_2O$ spraying, with genotype 7 recording a seven-fold concentration increase (Figure 2a,b). Regarding the Zn concentration in the grains, genotypes 3, 7, 14, and 18 receiving $ZnSO_4 \cdot 7H_2O$ showed the highest concentrations in both crops, about twice as high as treatments without Zn fertilization. No significant differences were observed in Zn concentration in the grains among genotypes that did not receive $ZnSO_4 \cdot 7H_2O$ spraying (Figure 2c,d). There were no differences in the concentration of nutrients, including Fe (Table S2).

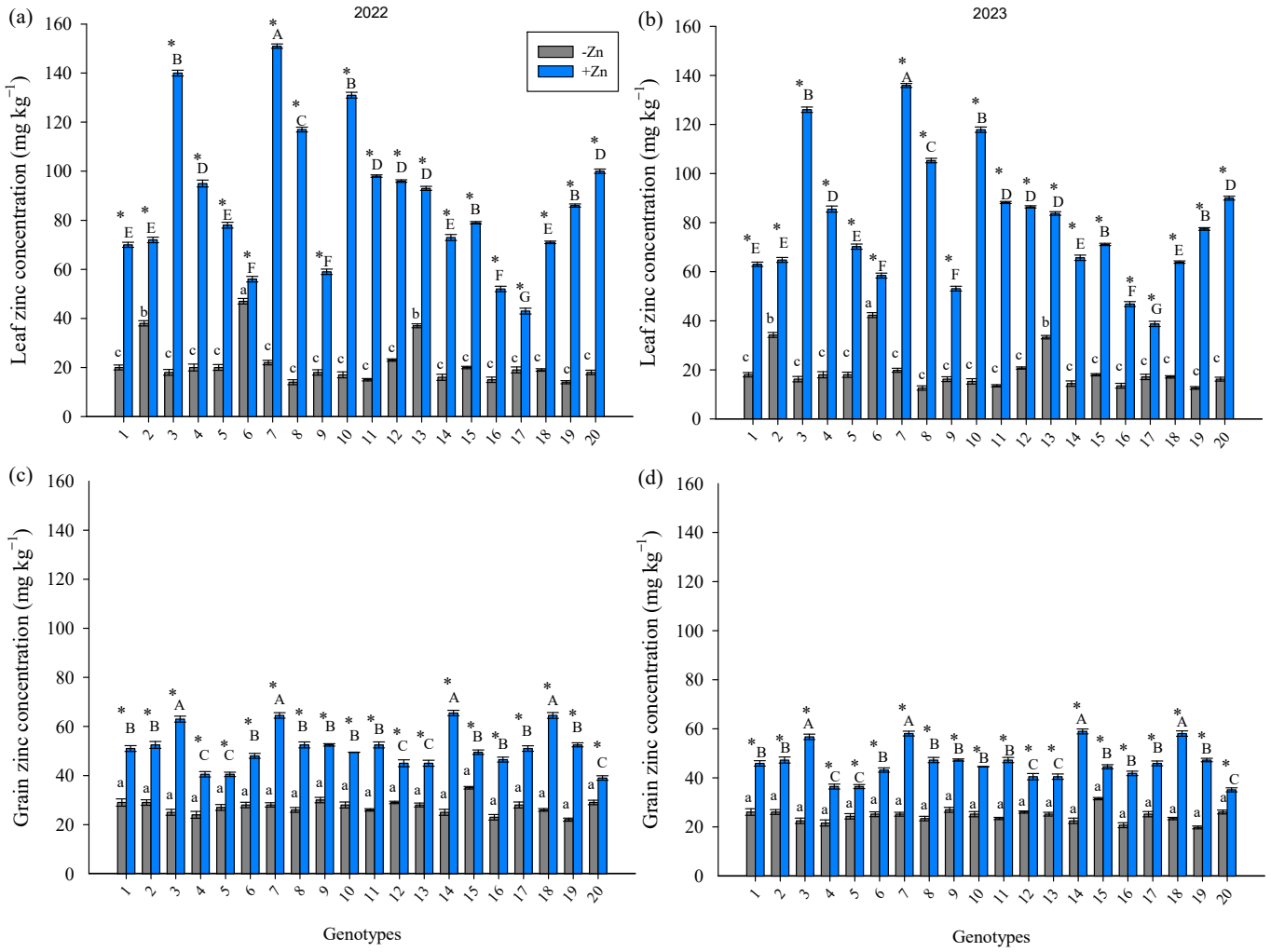

Figure 2. Leaf and grain Zn concentration for the first (**a**–**c**) and second (**b**–**d**) growing seasons of twenty cowpea genotypes in response to the application of $ZnSO_4 \cdot 7H_2O$. Error bars represent the standard error of the mean (n = 3). Different letters denote significant differences between means as determined by the Scott–Knott test ($p \leq 0.05$). Uppercase letters compare genotypes with Zn application, while lowercase letters compare genotypes without Zn application. * indicates a significant difference between means of the same genotype with and without Zn application according to the Scott–Knott test ($p \leq 0.05$).

The results of photosynthesis followed a pattern similar to yield data, where genotypes with $ZnSO_4 \cdot 7H_2O$ addition showed superior performance in both crops (Figure 3). Remarkably, genotype 7, which received foliar $ZnSO_4 \cdot 7H_2O$ spraying, demonstrated the best results in both crops. This genotype showed a two-fold increase in net photosynthesis rate (Figure 3a,b) and stomatal conductance (Figure 3c,d) compared to the same genotype without $ZnSO_4 \cdot 7H_2O$ application, and transpiration was also significantly increased with $ZnSO_4 \cdot 7H_2O$ application (Figure 3e,f).

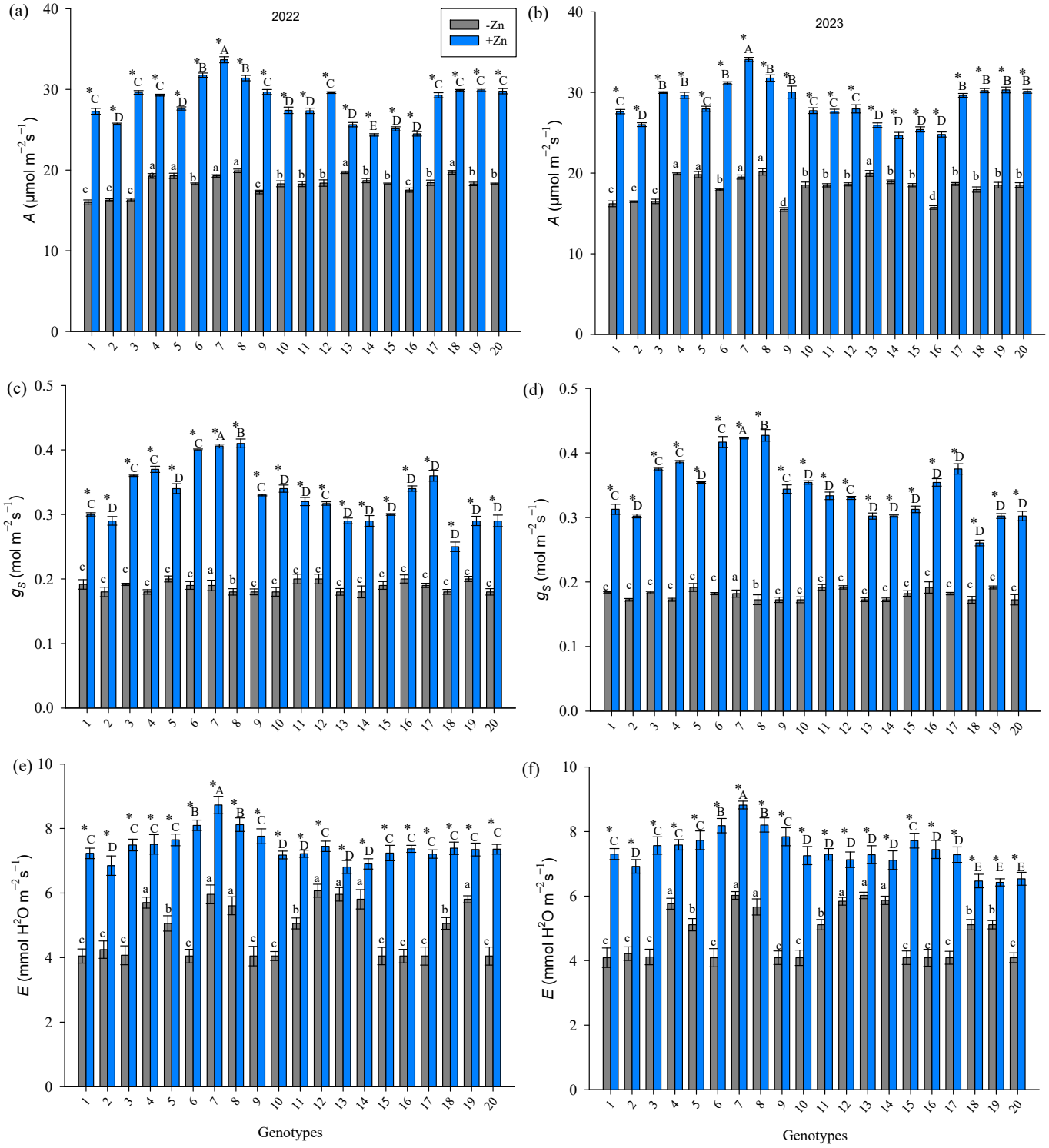

Figure 3. Net photosynthesis rate (A), stomatal conductance (g_s), and transpiration (E) for the first (**a**,**c**,**e**) and second (**b**,**d**,**f**) growing seasons of twenty cowpea genotypes in response to the application of $ZnSO_4 \cdot 7H_2O$. Error bars represent the standard error of the mean (n = 3). Different letters denote significant differences between means as determined by the Scott–Knott test ($p \leq 0.05$). Uppercase letters compare genotypes with Zn application, while lowercase letters compare genotypes without Zn application. * indicates a significant difference between means of the same genotype with and without Zn application according to the Scott–Knott test ($p \leq 0.05$).

The concentration of sucrose and total sugar in cowpea grains showed a significant increase with $ZnSO_4 \cdot 7H_2O$ application in both crops (Figure 4). Specifically, genotypes 3, 5, 7, 10, 11, and 19 exhibited the highest concentrations of sucrose and total sugar in both crops when treated with Zn. On average, between the two crops, genotypes receiving Zn showed an increase of 44% in sucrose concentration and an increase of 70% in total sugar compared to genotypes that did not receive $ZnSO_4 \cdot 7H_2O$. Additionally, $ZnSO_4 \cdot 7H_2O$ application also promoted an increase in the total levels of free amino acids in all evaluated genotypes, being two-fold higher in cowpea grains subjected to $ZnSO_4 \cdot 7H_2O$ application compared to those without $ZnSO_4 \cdot 7H_2O$ addition (Table 1).

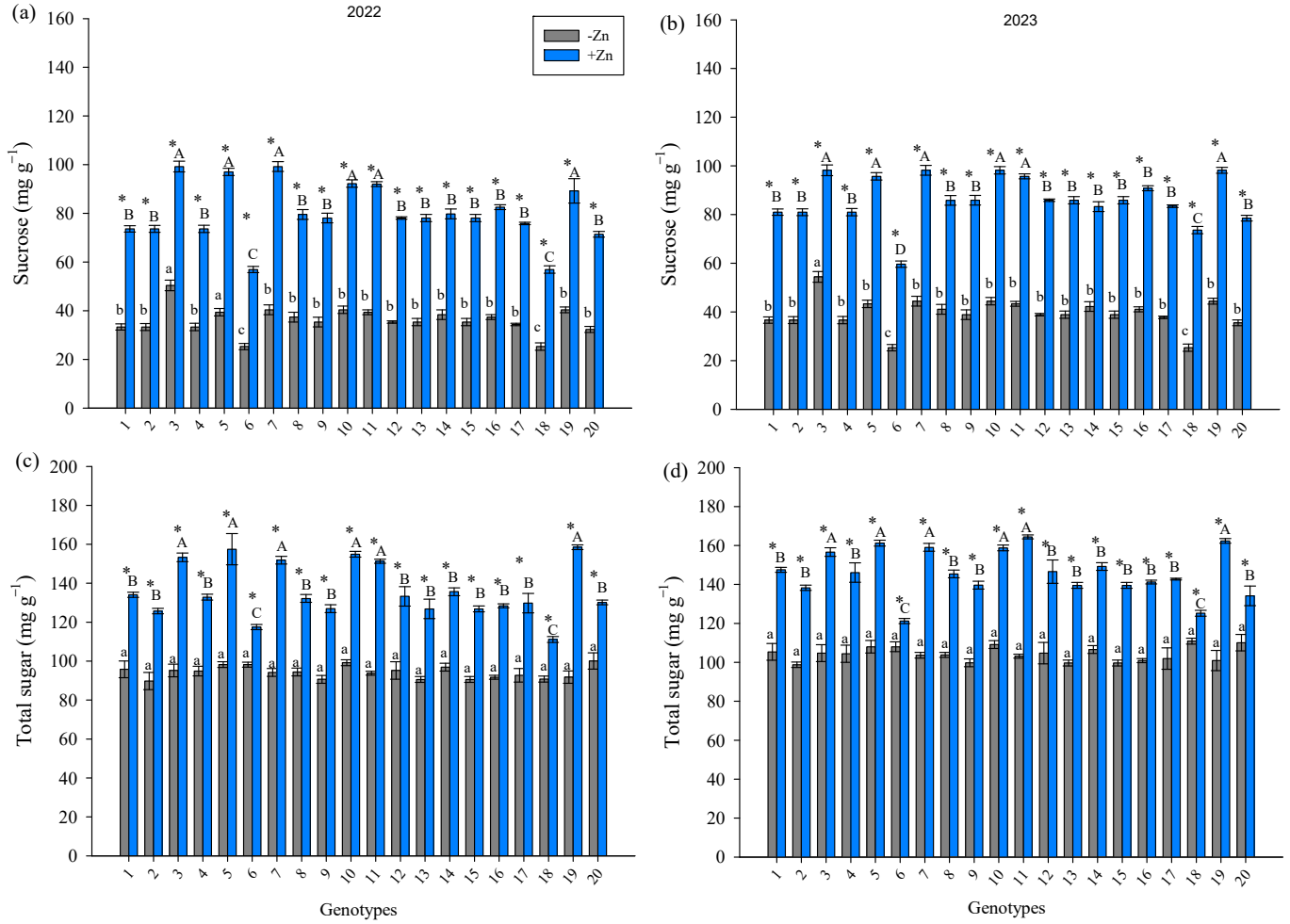

Figure 4. Concentration of sucrose and total sugar for the first (**a**–**c**) and second (**b**–**d**) growing seasons of twenty cowpea genotypes in response to the application of $ZnSO_4 \cdot 7H_2O$. Error bars represent the standard error of the mean (n = 3). Different letters denote significant differences between means as determined by the Scott–Knott test ($p \leq 0.05$). Uppercase letters compare genotypes with Zn application, while lowercase letters compare genotypes without Zn application. * indicates a significant difference between means of the same genotype with and without Zn application according to the Scott–Knott test ($p \leq 0.05$).

There was an increase in storage protein concentration in all evaluated genotypes in response to $ZnSO_4 \cdot 7H_2O$ application during both crop seasons (Figures 5 and 6). However, a complex interaction was observed between $ZnSO_4 \cdot 7H_2O$ application and genotypes, which affected different protein fractions. For albumin, the genotypes showing higher concentrations with $ZnSO_4 \cdot 7H_2O$ application were as follows: in the 2022 crop—1, 2, 3, 4, 10, 11, 12, 15, 16, 17, 18, 19, and 20 (Figure 5a); and in the 2023 crop—1, 2, 3, 10, 11, 12, 15, 16, 17, 18, 19, and 20 (Figure 5b). Regarding globulin, the genotypes demonstrating

higher concentrations in response to $ZnSO_4 \cdot 7H_2O$ application were 1, 9, 15, 16, and 20 in both crops (Figure 5c,d). For prolamin, the genotypes with higher concentrations after $ZnSO_4 \cdot 7H_2O$ application in both crops were 1, 2, 3, 6, 9, 10, 11, 12, 13, 15, 17, and 20 (Figure 6a,b). In the case of glutelin, genotype 3 stood out with a higher concentration compared to other genotypes in both crops and recorded a 35% increase compared to the same genotype without $ZnSO_4 \cdot 7H_2O$ application (Figure 6c,d). The results showed genotypic variation, where genotypes did not follow a uniform pattern. For example, among plants with $ZnSO_4 \cdot 7H_2O$ fertilization, some genotypes showed high yields and low accumulation of reserve proteins, while others demonstrated low yield and low protein accumulation. On the other hand, there were cases where genotypes with low yield exhibited high levels of protein accumulation, just as some genotypes with high yield also showed high protein levels.

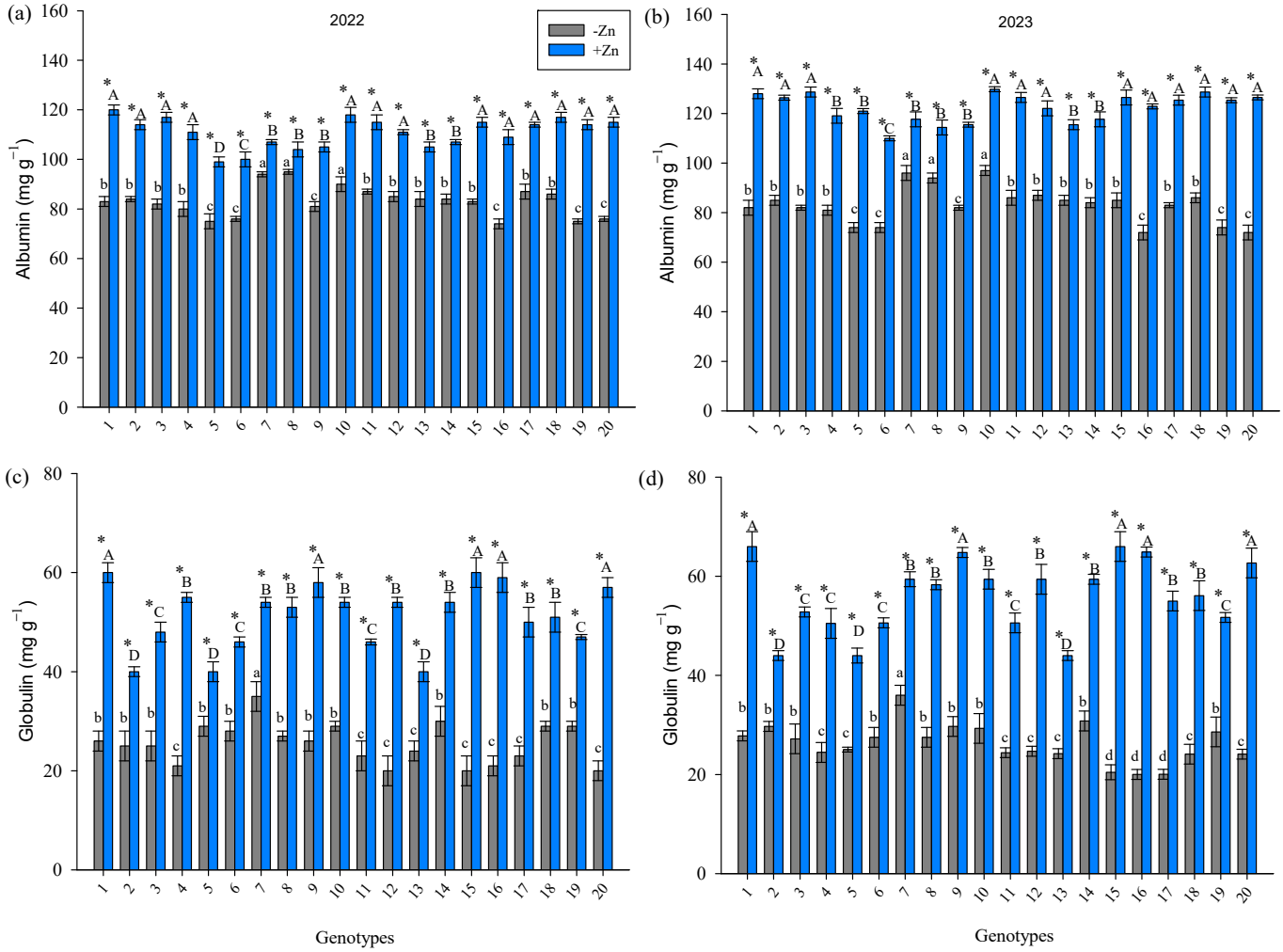

Figure 5. Concentration of albumin and globulin for the first (**a**–**c**) and second (**b**–**d**) growing seasons of twenty cowpea genotypes in response to application of $ZnSO_4 \cdot 7H_2O$. Error bars represent the standard error of the mean (n = 3). Different letters denote significant differences between means as determined by the Scott–Knott test ($p \leq 0.05$). Uppercase letters compare genotypes with Zn application, while lowercase letters compare genotypes without Zn application. * indicates a significant difference between means of the same genotype with and without Zn application according to the Scott–Knott test ($p \leq 0.05$).

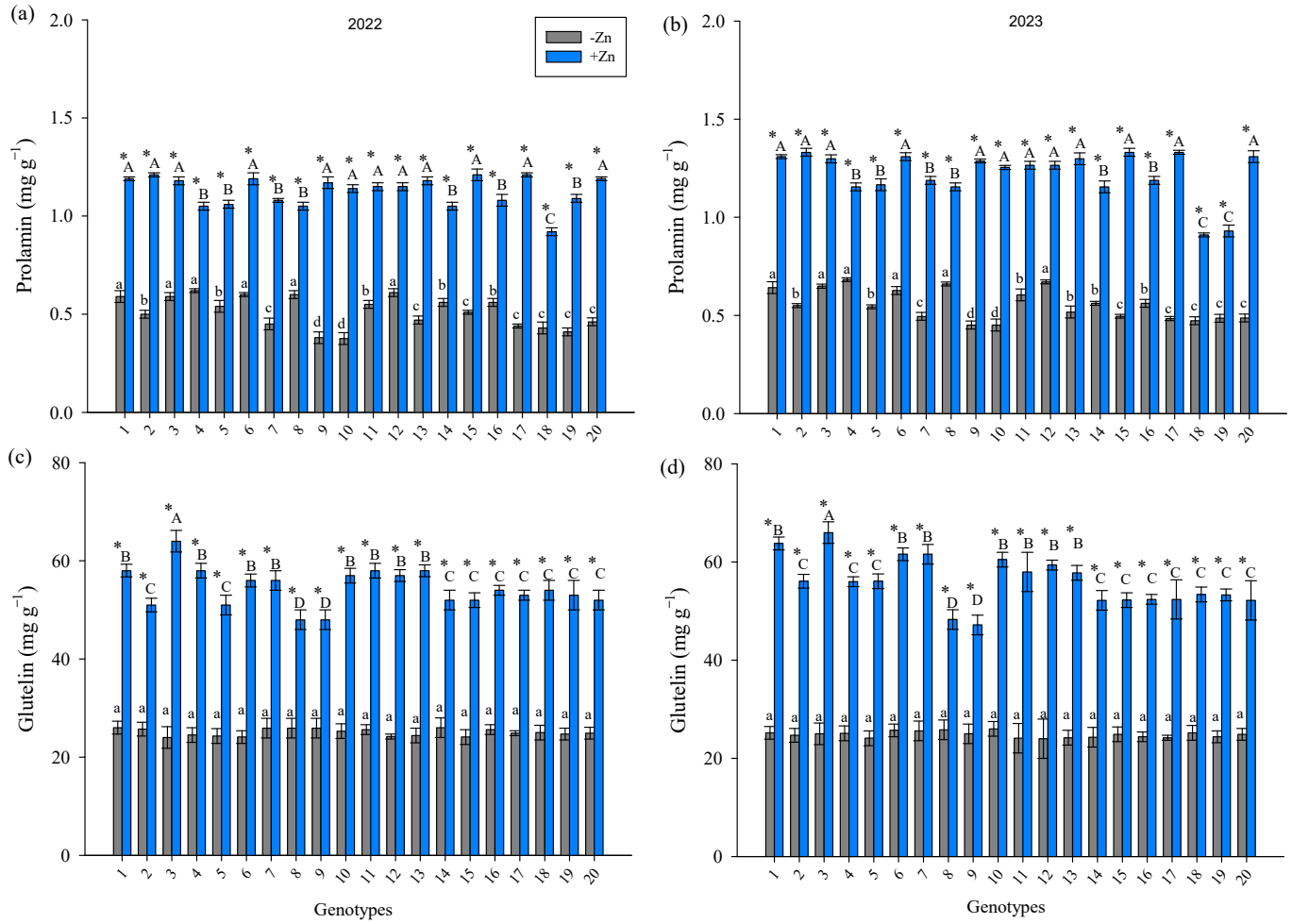

Figure 6. Concentration of prolamin and glutelin the first (**a**–**c**) and second (**b**–**d**) years of twenty cowpea genotypes in response to the application of $ZnSO_4 \cdot 7H_2O$. Error bars represent the standard error of the mean (n = 3). Different letters denote significant differences between means as determined by the Scott–Knott test ($p \leq 0.05$). Uppercase letters compare genotypes with Zn application, while lowercase letters compare genotypes without Zn application. * indicates a significant difference between means of the same genotype with and without Zn application according to the Scott–Knott test ($p \leq 0.05$).

Table 1. Concentrations of total free amino acids (mg kg^{-1}) of twenty cowpea genotypes.

ID	Genotype	−Zn	+Zn
1	MNC11-1013E-33	1.14	3.95
2	MNC11-1013E-16	1.37	3.22
3	MNC11-1013E-15	1.04	3.14
4	MNC11-1013E-35	1.58	3.85
5	MNC11-1018E-17	1.04	3.64
6	MNC11-1019E-8	1.08	3.45
7	MNC11-1019E-12	1.56	3.92
8	MNC11-1019E-46	1.34	3.8
9	MNC11-1020E-16	1.16	3.32
10	MNC11-1022E-58	1.23	3.07
11	MNC11-1024E-1	1.42	3.49
12	MNC11-1026E-15	1.29	3.96
13	MNC11-1022E-19	1.48	3.36

Table 1. *Cont.*

ID	Genotype	−Zn	+Zn
14	MNC11-1031E-5	1.5	3.61
15	MNC11-1031E-11	1.33	3.58
16	MNC11-1034E-2	1.47	3.41
17	MNC11-1052E-3	1.36	3.01
18	BRS Pajéu	1.21	3.86
19	BRS Marataoã	1.38	3.17
20	BRS Rouxinol	1.29	3.08
	Average	1.34 b	3.47 a

Different letters indicate differences between means according to the Scott–Knott test ($p \leq 0.05$).

4. Discussion

Foliar Zn fertilization has been shown to have a significant impact on the yield and quality of cowpea grains. Zinc is a micronutrient that plays an important role in several metabolic pathways and physiological processes in plants. It acts as a cofactor in many enzymes involved in photosynthesis, carbohydrate metabolism, and protein synthesis [28–30]. For example, Zn is essential for the activity of carbonic anhydrase (EC 4.2.1.1), an enzyme that facilitates the transfer of CO_2/HCO_3 for photosynthetic carbon (C) fixation [28,31]. Furthermore, Zn is also involved in the regulation of photosynthate metabolism, which affects the activity of enzymes such as superoxide dismutase (EC 1.15.1.1) and d-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) [28,32]. Therefore, the addition of Zn can increase the efficiency of photosynthesis and, consequently, the yield of cowpeas.

The results of photosynthesis followed a pattern similar to yield data. Genotypes that exhibited higher yield due to $ZnSO_4 \cdot 7H_2O$ spraying also showed higher rates of net photosynthesis, stomatal conductance, and transpiration. This is explained by the fact that Zn is an essential component of various enzymes involved in photosynthesis, such as carbonic anhydrase and RuBisCO (ribulose 1,5-disphosphate carboxylase, EC 4.1.1.39), which facilitates CO_2 fixation and increases chlorophyll production efficiency [33,34]. Additionally, Zn is involved in carbohydrate metabolism and cellular respiration, enhancing the activity of enzymes such as pyruvate dehydrogenase (EC 1.2.4.1) and isocitrate dehydrogenase (EC 1.1.1.42). This results in more efficient cellular respiration [35–37]. Regarding stomatal conductance, Zn influences the regulation of ion movement across cell membranes, affecting stomatal opening and facilitating the entry of CO_2 and the exit of water vapor during transpiration [38]. Similar results to ours were observed by other authors in different crops. Liu et al. [29], when evaluating the yield of maize and wheat as a function of Zn application, concluded that increases in the yield of these crops were mainly achieved through improvements in photosynthetic rate and leaf area index resulting from foliar Zn spraying. Esfandiari et al. [39], assessing the impact of foliar Zn application on grain production, also observed higher yield and related this improvement to increased enzymatic activity, photosynthetic rate, and translocation of photoassimilates to grains resulting from Zn application. Yeboah et al. [40] observed that the addition of Zn to bean plants had a beneficial effect on chlorophyll content and stomatal conductance, resulting in a higher yield.

The observation of higher concentrations of Zn in the leaves and grains of cowpeas in genotypes subjected to Zn addition confirms the effectiveness of agronomic biofortification in this context. However, there was observed genotypic variability in the response, with some genotypes proving to be superior to others. Zinc concentrations in cowpea grains from plants sprayed with Zn ranged from 38 to 62 mg kg^{-1}, on average, over the two crop seasons (Figure 2c,d). These values are close to those (47–59 mg kg^{-1}) found by Silva et al. [3], when evaluating Zn biofortification in 29 cowpea genotypes. However, a greater variation in Zn concentrations in grains was observed in our work. The variation in the magnitude of response among different cowpea genotypes highlights the importance of considering the specific genetic characteristics of each genotype. In agreement with the

observations of Oliveira et al. [41], Rocha et al. [42], and Silva et al. [3], this genotypic variation is expected due to the high morphological and genetic diversity of cowpea genotypes. Some genotypes may be more efficient in Zn uptake and translocation than others, which can directly influence the concentration of this micronutrient in leaves and grains [28].

In addition to the increase in Zn concentration in the grains of genotypes that received $ZnSO_4 \cdot 7H_2O$ spraying, there was an increase in the concentration of sucrose, total sugar, and total amino acids in cowpea grains in both seasons evaluated across all genotypes. It is plausible that the addition of Zn may have directly influenced the activities of enzymes involved in the synthesis and metabolism of carbohydrates and amino acids in cowpea grains [36,43]. Among these enzymes, sucrose synthase and invertase stand out, responsible for the synthesis and decomposition of sucrose, respectively [44]. With the presence of Zn, it is possible that the activity of these enzymes is increased, promoting greater sucrose synthesis in the grains. Additionally, the observed increase in photosynthetic rate and stomatal conductance with Zn addition may have contributed to an increase in C assimilation by the plants. This, consequently, may have triggered the observed increase in sucrose concentration, total sugar, and total amino acids in cowpea grains after Zn application. The positive effects of Zn on sugars, sucrose, and total amino acids were also observed in previous studies. In some cowpea genotypes, Zn increased total sugar levels in grains [3]. In cabbage cv. Bronco, Zn application increased the concentration of total amino acids [45]. In common beans, with foliar application of Zn at the same dose used in our study (600 g ha^{-1}), there was an increase in the concentrations of total amino acids, sucrose, and total sugars in grains [6].

The application of $ZnSO_4 \cdot 7H_2O$ increased the concentration of storage proteins in the grains in all genotypes evaluated during the two seasons (Figures 5 and 6). This increase can be attributed to Zn being a cofactor of several enzymes involved in protein synthesis, including those participating in the transcription and translation of genes responsible for protein production [28,46,47]. Additionally, Zn is also involved in the regulation of gene expression related to protein synthesis, which can directly influence transcription and translation processes in ribosomes [28]. This gene regulation may increase the expression of genes responsible for the synthesis of storage proteins in cowpea grains. Another important aspect is the role of Zn in the stability and integrity of protein structures. Zinc acts as a structure stabilizer in some proteins, ensuring their correct three-dimensional conformation and, consequently, their functionality [47,48]. The increase in protein concentration in cowpea grains after Zn application can also be attributed to the effect of this micronutrient on the enzymatic pathways responsible for N assimilation [3,5].

The Zn source used in the study ($ZnSO_4 \cdot 7H_2O$) contains sulfur (S), an element that plays a role in protein synthesis [49]. This may have influenced the nutritional quality of cowpea grains. Sulfur is known for its role in the formation of amino acids and proteins, and its presence may have contributed to the observed results in protein content in the grains [49]. Although our study primarily focused on the effects of Zn, the additional benefits of sulfur cannot be disregarded. We acknowledge that further research is needed to understand the extent of these effects.

In all evaluated attributes, genotypic variation in response to Zn addition was observed, highlighting the complexity of interactions between different cowpea genotypes and Zn fertilization. For example, genotype 7 (MNC11-1019E-12) exhibited the highest yield, greater photosynthesis, and Zn concentration in both leaves and grains with Zn application in the two seasons. However, this genotype did not show the highest protein concentration. The lack of a uniform response pattern, particularly concerning protein concentrations in grains and yield among genotypes, suggests the influence of multiple factors, both genetic and environmental, on the phenotypic expression of plants. Different genotypes may exhibit varied levels of efficiency in Zn utilization, which can affect their response to the addition of this micronutrient. Additionally, specific environmental factors for each genotype may also influence their response to Zn addition [28].

Based on all observed results, the significant contribution of agronomic Zn biofortification to improving the nutritional quality of cowpea crops is emphasized. The addition of Zn not only resulted in an increase in the concentration of this micronutrient in grains but also promoted an increase in other important compounds, such as total amino acids and storage proteins. These benefits are of great importance, especially in regions where Zn deficiency is a public health concern.

5. Conclusions

The results of this study clearly demonstrate the positive effects of agronomic biofortification with Zn on the nutritional quality and yield of cowpeas. The addition of Zn resulted not only in an increase in the concentration of this micronutrient in grains but also promoted an increase in other important compounds, such as total amino acids, sucrose, total sugars, and storage proteins. This highlights the importance of biofortification as a strategy to improve food security and nutrition, especially in regions where Zn deficiency is a public health concern. Additionally, the genotypic variation observed in response to Zn addition underscores the need to consider different genotypes and environmental factors when developing biofortification strategies. In the context of the United Nations Sustainable Development Goals (https://www.undp.org/sustainable-development-goals, accessed on 1 February 2024), agronomic biofortification emerges as a promising tool to achieve goals related to food security, health, and well-being. Continuing research in this area is essential to expand access to more nutritious foods and promote more sustainable and resilient agriculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14060911/s1, Table S1: Identity, commercial class and characteristics of cowpea genotypes used in this study based on commercial subclass, maturation cycle and breeding method of obtention; Table S2: Macronutrient and micronutrient concentrations in leaves of twenty cowpea genotypes collected at full bloom.

Author Contributions: C.F.O. and M.G.S. set up and conducted the experiment, performed the laboratory analyses, and conceptualized and wrote the manuscript. G.N.S. and K.R.D. performed statistical analyses and aided in the results and discussion writing. M.d.M.R., A.R.R., F.H.S.R. and J.L. provided support in the experiment conduction, laboratory analyses, and writing. E.F.S. planned, conceptualized, and coordinated the project and validated the writing and analyses. All authors have read and agreed to the published version of the manuscript.

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