Plant Physiology and Biochemistry 162 (2021) 378–387

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Agronomic biofortification of cowpea with zinc: Variation in primary metabolism responses and grain nutritional quality among 29 diverse genotypes

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ARTICLE INFO

Keywords:
Vigna unguiculata (L.) Walp
Sugars
Ureides
Amino acids
Phytic acid
Phytate
Protein

ABSTRACT

Dietary zinc (Zn) deficiency is widespread globally, and is particularly prevalent in low- and middle-income countries (LMICs). Cowpea (*Vigna unguiculata* (L.) Walp) is consumed widely in LMICs due to its high protein content, and has potential for use in agronomic biofortification strategies using Zn. This study aimed to evaluate the effect of Zn biofortification on grain nutritional quality of 29 cowpea genotypes. Zn application did not increase cowpea yield. In 11 genotypes sucrose concentration, in 12 genotypes total sugar concentration, and in 27 genotypes storage protein concentration increased in response to Zn supply. Fifteen genotypes had lower concentrations of amino acids under Zn application, which are likely to have been converted into storage proteins, mostly comprised of albumin. Phytic acid (PA) concentration and PA/Zn molar ratio were decreased under Zn application. Six genotypes increased shoot ureides concentration in response to Zn fertilization, indicating potential improvements to biological nitrogen fixation. This study provides valuable information on the potential for Zn application to increase cowpea grain nutritional quality by increasing Zn and soluble storage protein and decreasing PA concentration. These results might be useful for future breeding programs aiming to increase cowpea grain Zn concentrations through biofortification.

1. Introduction

More than half of the world's population consumes micronutrients at concentrations lower than their daily minimal requirements (Masuda et al., 2020). Human nutrient deficiencies, known as "hidden hunger", affect about 2 billion people worldwide (Gödecke et al., 2019). Hidden hunger affects all classes of society, but infants, babies and pregnant women are most vulnerable. One third of all women at reproductive age are anemic, being more vulnerable to malnutrition (Kumar and Pandey, 2020). Zinc (Zn) deficiency is a global health problem, being the second most common nutrient deficiency worldwide (Haider et al., 2020). Is estimated that 17% of worldwide population suffers from inadequate Zn intake based on supply (Kumssa et al., 2015). The prevalence of Zn deficiency is greater in LMICs (Joy et al., 2014).

Biofortification of crops is a method to increase nutritional value of edible plants using agronomic practices, transgenic tools and breeding programs (Lividini et al., 2018). Agronomic biofortification through application of fertilizers has been shown to be a good potential strategy to increase Zn concentration in edible crops, with the main focus on cereals (Manzeke et al., 2014; Kumar et al., 2019). Studies of agronomic biofortification to increase Zn concentration are already established in many legume crops (Kumar and Pandey, 2020), including chickpea (Grewal et al., 2020), common bean (Philipo et al., 2020), mungbean (Haider et al., 2020), and cowpea (Manzeke et al. 2017, 2020).

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https://doi.org/10.1016/j.plaphy.2021.02.020
Received 29 January 2021; Accepted 18 February 2021
Available online 21 February 2021

Cowpea (*Vigna unguiculata* (L.) Walp), is an important source of protein, mostly to populations in LMICs of Asia, Africa and South America (Manzeke et al., 2017; Silva et al., 2019). Cowpea grains are widely variable in color, texture, size, and chemical composition (Rocha et al., 2017). The content of protein in cowpea grains is higher than common bean (Teka et al., 2020). Since Zn plays a role in nitrogen metabolism, Zn application could be related to protein concentration in cowpea grains (Moura et al., 2012). Thus, Zn biofortification in cowpea provides potential to increase Zn content in grains, which might further benefit human nutrition.

Studies on genotypic effects in cowpea in response to Zn fertilization have not yet been reported. Potential genotypic variation could be better explored in terms of micronutrients accumulation in grains, and improved grain quality, aiming to benefit human health by combating hidden hunger. The effects of Zn fertilization on other potential traits which contribute to cowpea grain quality could also be explored. These include such phytic acid (PA) concentration. Phytic acid is the major form of phosphorus storage in seeds, forming complex stable components with Zn, leading to a decrease in Zn bioavailability in the gastrointestinal tract (Grewal et al., 2020). Other quality traits include sugars and proteins.

The aim of this study was to determine the effect of Zn application on grain nutritional quality and Zn partitioning and accumulation in 29 cowpea genotypes. Therefore, in addition to Zn, the following grain quality properties were measured: total sugar, sucrose, amino acids, storage proteins and phytic acid concentration. Discovering cowpea genotypes showing high capacity for Zn uptake and accumulation, high soluble proteins, and decreased phytic acid concentration in grains is a good starting point for future breeding programs.

2. Material and methods

2.1. Experiment design

The experiment was carried out under greenhouse conditions at São Paulo State University (UNESP), Tupã, São Paulo State, Brazil. Twenty-nine genotypes of cowpea were cultivated in response to absence (control) and 25 mg kg^{-1} of Zn applied as zinc sulfate heptahydrate. All seeds of cowpea genotypes used in this study were cultivated and obtained from Brazilian Agricultural Research Corporation (EMBRAPA) germplasm bank. Information regarding genotypes provenance, maturation cycle, and geographical recommendation are listed in Table 1. The experimental design was completely randomized, with three replications for each genotype totaling 174 pots. In November 2016, the soil was collected from UNESP experimental farm and sieved 4 mm mesh to fill 5 kg pots. The soil was classified as Oxisol and chemical properties were as follows: pH ($CaCl_2$ 0.01 M) 4.6; phosphorus (resin): 6 mg dm^{-3}, sulfur: 3 mg dm^{-3} (calcium phosphate), boron (hot water): 0.07 mg dm^{-3}, copper (diethylene triamine pentacetic acid - DTPA): 0.5 mg dm^{-3}, iron (DTPA): 11 mg dm^{-3}, manganese (DTPA): 12 mg dm^{-3}, zinc (DTPA): 0.2 mg dm^{-3}; potassium (resin): 0.9 $mmol_c$ dm^{-3}, calcium (resin): 5 $mmol_c$ dm^{-3}, magnesium (resin): 3 $mmol_c$ dm^{-3}, H + Al (SMP buffer): 16 $mmol_c$ dm^{-3}, cation exchange capacity: 24.9 $mmol_c$ dm^{-3} and base saturation: 36% (Raij et al., 1997).

Prior to the experiment, 1.25 g of lime, 0.46 g single superphosphate, and 0.17 g KCl was applied per pot in order to neutralize the soil pH and provide proper fertility for cowpea plants according to recommendation for bean plants (Quaggio and Raij, 1997). The pots filled with soil were kept at incubation for 30 days before sowing. The seeds were inoculated with a peat inoculum specific for cowpea (Strain SEMIA 6462, BIOMAX, 2.0 x 10^9 colony forming units g^{-1}, São Joaquim da Barra city, Brazil) at 8 g kg^{-1} of seed. Prior the inoculation, a 10% sugar solution was used to dissolve the inoculum, and then, the solution was added and mixed with the seeds. On December 29 2016, sowing was performed, with

Table 1
Identity and characteristics of cowpea genotypes used in this study based on grain color, maturation cycle and geographical recommendation in Brazil.

ID	Genotype	Grain color	Maturation Cycle*	Origin	Method of obtention	Geographical recommendation**
1	BR 17 - Gurguéia	Brown	Early Medium	Conventional breeding	Crossbreeding	A**
2	BR 3-Tracuateua	White	Early	Conventional breeding	Crossbreeding	"São Paulo" State
3	BRS Aracê	Green	Early Medium	Conventional breeding	Crossbreeding	B**
4	BRS Cauamé	White	Early	Conventional breeding	Crossbreeding	A
5	BRS Guariba	White	Early	Conventional breeding	Crossbreeding	A
6	BRS Itaim	White	Early	Conventional breeding	Crossbreeding	B
7	BRS Juruá	Green	Early medium	Conventional breeding	Crossbreeding	B
8	BRS Marataoã	Brown	Early medium	Conventional breeding	Crossbreeding	A
9	BRS Milênio	White	Early medium	Conventional breeding	Individual Plants selection after progeny test	"Pará" State
10	BRS Novaera	White	Early	Conventional breeding	Crossbreeding	A
11	BRS Pajeú	Brown	Early Medium	Conventional breeding	Crossbreeding	North and Northeast regions
12	BRS Potengi	White	Early	Conventional breeding	Crossbreeding	North and Northeast regions
13	BRS Rouxinol	Brown	Early Medium	Conventional breeding	Crossbreeding	"Bahia" State
14	BRS Tumucumaque	White	Early	Conventional breeding	Crossbreeding	A
15	BRS Urubuquara	White	Early Medium	Conventional breeding	Individual Plants selection after progeny test	"Para" State
16	BRS Xiquexique	White	Early Medium	Conventional breeding	Crossbreeding	A
17	California CB-5	White	Early	Conventional breeding	Crossbreeding	California State (USA)
18	Inhuma	Brown	Early Medium	Landrace	Field collection from farmers	A
19	MNC01-631F-20-5	Brown	Early Medium	Conventional breeding	Crossbreeding	North and Northeast regions
20	MNC04-769F-62	Brown	Early	Conventional breeding	Crossbreeding	A
21	MNC04-782F-108	Brown	Early Medium	Conventional breeding	Crossbreeding	A
22	MNC04-792F-143	Brown	Early	Conventional breeding	Crossbreeding	A
23	MNC04-792F-146	Brown	Early	Conventional breeding	Crossbreeding	A
24	MNC04-795F-158	Brown	Early Medium	Conventional breeding	Crossbreeding	A
25	Patativa	Brown	Early Medium	Landrace	Field collection from farmers	"Ceará" State
26	Paulistinha	Brown	Early Medium	Landrace	Field collection from farmers	"Piauí" and "Ceará" states
27	Pingo de Ouro-1-2	Brown	Early Medium	Conventional breeding	Individual Plants selection after progeny test	A
28	Pingo de Ouro-2	Brown	Early Medium	Conventional breeding	Individual Plants selection after progeny test	Northeast Region
29	Pretinho	Black	Medium	Landrace	Field collection from farmers	A

"Early" maturation cycle last for 60–65 days; "Early Medium" maturation cycle lasts for 70–75 days, and "Medium" maturation cycle lasts for 75–80 days. ** "A" for genotypes recommended for North and Northeast regions, and "Mato Grosso" and "Mato Grosso do Sul" states; "B" For genotypes recommended for "Pará", "Roraima", "Mato Grosso", "Piauí", "Tocantins", "Maranhão", "Bahia" and Sergipe States.

emergence at 7 days after sowing. Commercial urea was diluted in deionized water and applied 25 mL to each pot at the concentration equivalent to 0.10 g pot^{-1} at 20 days after emergence (DAE) to enhance nitrogen metabolism and plant growth. A custom computerized irrigation system was used to provide irrigation. At 29, 36 and 61 DAE application of urea (0.22 g pot^{-1}), KCl (0.17 g pot^{-1}), and single superphosphate (0.45 g pot^{-1}) was applied in all pots. Application of 25 mg dm^{-3} of Zn was applied at 45 DAE. A stock solution of zinc sulfate heptahydrate was prepared in distilled water and treated pots received 25 mL of solution containing Zn equivalent to 25 mg dm^{-3}.

Harvest was performed across a series of days for each genotype according to the pod's maturity. The plant material was separated into three parts: grains, leaves + stems and roots. The separated material was dried in an oven at 65 °C for 72 h to a constant mass to measure the dry weight (DW) $plant^{-1}$ of grains, leaves + stems and roots. Then, the material was homogenized in a Wiley mill for further chemical and biochemical analysis.

2.2. Zinc measurements

Zinc analysis were performed according to Thomas et al. (2016). Samples of roots, leaves + stems, and grains were weighed to approximately 0.250 g and digested in 2 mL of HNO_3 (70%), 1 mL of Milli-Q water and 1 mL of H_2O_2 (30%). The digestion was performed in a microwave system with a 48-vessel MF50 rotor (Anton Paar GmbH, Graz) at 140 °C and 2 MPa for 45 min. The concentrations of Zn were obtained using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany). Zinc concentration was expressed in mg kg^{-1} DW.

Zinc partitioning to shoots and grains were estimated according to Abichequer and Bohnen (1998) following the equations:

$$Zn\ partitioning\ to\ shoot(\%) = \frac{(B \times E) + (C \times F)}{(A \times D) + (B \times E) + (C \times F)} \quad (1)$$

$$Zn\ partitioning\ to\ grains(\%) = \frac{(C \times F)}{(A \times D) + (B \times E) + (C \times F)} \quad (2)$$

In which:

A – Root dry weight (kg $plant^{-1}$)
B – Leaves + stems dry weight (kg $plant^{-1}$)
C – Grain dry weight (kg $plant^{-1}$)
D – Root Zn concentration (mg kg^{-1})
E – Leaves + stems Zn concentration (mg kg^{-1})
F – Grains Zn concentration (mg kg^{-1})

2.3. Extraction for sugars, amino acids and ureides

Total sugar, sucrose and total amino acids concentrations were determined from 0.5 g of milled grain samples, and ureides (allantoin and allantoic acid) were determined from 0.3 g of milled leaves + stems samples extracted in 10 mL of MCW solution (60% methanol, 25% chloroform, and 15% water) and prepared to analysis according to Bielesk and Turner (1996). In a 15 mL conical bottom polystyrene tube (Kasiv, K19-0015, China), the material was homogenized by vortexing and kept in the refrigerator for 24 h. Afterwards, the tubes were centrifuged at 10,000 rpm for 10 min at 4 °C. To another clean tube was added 5 mL of MCW extract supernatant, 1.25 mL of chloroform and 1.875 mL of water. After a waiting period of 48 h for phases separation, aliquots from the hydrophilic portion of the extract were used to total sugar, sucrose, amino acid, total ureides, allantoin and allantoic acid determination.

2.4. Sucrose and total sugar concentration

Sucrose was quantified according to Van Handel (1968), whereby 20 μL of hydrophilic portion of MCW extract, 500 μL of 30% KOH and 2 mL of concentrated H_2SO_4 was added to a glass tube. The mixture was homogenized by vortexing and oven-dried at 100 °C for 10 min. After cooling to room temperature, the absorbance at 490 nm was read using a spectrophotometer (SP-220, bioespectroTM). The results for sucrose were expressed as mg g^{-1} DW.

Total sugar was quantified according to the protocols in Dubois et al. (1956), whereby 20 μL of hydrophilic portion of MCW extract, 500 μL of 5% phenol and 2 mL of concentrated H_2SO_4 was added to a glass tube. The mixture was homogenized by vortexing, and after cooling to room temperature, the absorbance at 490 nm was read using a spectrophotometer (SP-220, bioespectroTM). The results were expressed as mg g^{-1} DW. To quantify both sugar and sucrose, a standard sucrose curve was used.

2.5. Amino acids concentration

Amino acids were quantified according to Yemm et al. (1955), whereby 250 μL of hydrophilic portion of MCW extract, 500 μL 0.2 M sodium citrate, 200 μL ninhydrin 5% in ethylene glycol and 1 mL 0.0002 M KCN was added to a glass tube. The tubes were mixed by vortexing and heat at 100 °C for 15 min. Afterwards, the tubes were cooled with tap water for 10 min. Then, 1 mL of 60% ethanol was added into glass tubes, mixed by vortexing, the absorbance at 570 nm was read using a spectrophotometer (SP-220, bioespectroTM). A methionine standard curve was used to calculate the concentration of grain amino acids and the results were expressed as mg g^{-1} DW.

2.6. Storage proteins

The storage proteins were analyzed in four different fractions: albumin, globulin, prolamin and glutelin. The quantification of storage protein in each extract was performed according to Bradford (1976). A 0.25 g sample of grain was extracted using 5 mL of deionized H_2O in a 15 mL conical bottom polystyrene tube. The material was homogenized by vortexing and then centrifuged in a refrigerated centrifuge at 10,000 rpm for 5 min at 4 °C. Then, 20 μL of supernatant was added to 1 mL of Bradford solution, in 2 mL safe lock microcentrifuge tubes. The microtubes were mixed by vortexing, and then, the absorbance at 595 nm was read using a spectrophotometer (SP-220, bioespectroTM) for determination of albumin concentration. The H_2O extract was disposed, but the grains sample residue within the tube was kept. The aforementioned process was repeated in the same sample, replacing 5 mL of deionized H_2O by 5 mL of NaCl 5% to determine globulin, then replacing 5 mL of NaCl 5% by 5 mL of ethanol 60% to determine prolamin, and finally, replacing 5 mL of ethanol 60% by 5 mL of NaOH 0.4%. to determine glutelin. A bovine serum albumin standard curve was used to calculate the concentration of grains storage proteins and the results were expressed as mg g^{-1} DW (dry weight).

2.7. Phytic acid concentration

Phytic acid (PA) was determined according to Silva et al. (2019). Approximately 1.0 g DW of milled grain samples were weighed and placed into 50 mL conical tubes (SARSTEDT). To each tube, 20 mL of 0.66 M HCl was added, and tubes were put in a rotary shaker to shake overnight at 25 rpm. After this, 1 mL of the homogenized solution was transferred to 2 mL safe lock microtubes (Eppendorf, Hamburg, Germany) and centrifuged at 13,000 rpm for 10 min. After centrifugation, 0.5 mL of the supernatant was transferred to a new 2 mL safe lock microtubes and neutralized with 0.5 mL of 0.75 M NaOH. This extract was used to perform an enzymatic dephosphorylation reaction, in order to estimate PA concentration from the difference between total

phosphorus and free phosphorus assayed using the phytic acid (total phosphorus) Assay Kit (Megazyme) following the manufacturer's instructions. The absorbance of phosphomolybdate was read in 96 well plate readers (Thermo Scientific, Waltham, MA, USA) at 650 nm in an EL808 absorbance reader (BIOTEK). One phosphorus calibration curve was included in each plate. The Pi standard used to estimate de PA concentration was Oat flour control powder with an established concentration of 17,700 mg kg^{-1}. The results were expressed as mg g^{-1} DW. To estimate PA/Zn molar ratio, the PA concentration of each sample was divided by Zn concentration in each sample, converting both variables to mmol kg^{-1}.

2.8. Ureides concentration

Total ureides, allantoin and allantoic acid were determined according to Vogels and Van Der Drift (1970). To determinate allantoin, 250 μL of hydrophilic portion of MCW extract, 250 μL of NaOH 0.5 M and 20 μL of 0.33% phenylhydrazine were added to a glass tube. The mixture was homogenized by vortexing and oven-dried at 100 °C for 8 min. After cooling to room temperature, it was added 250 μL of HCl 0.65 N, the mixture was again homogenized by vortexing and oven-dried at 100 °C for 4 min. After another cooling to room temperature, it was added 250 μL of phosphate buffer solution 0.4 M, pH 7.0 and 250 μL of 0.33% phenylhydrazine. The mixture was homogenized and kept at room temperature by 5 min, then cooled in ice by 5 more minutes. Then, it was added 1.25 mL of 37% HCl and 250 μL of 1.65% $K_3Fe(CN)_6$. The mixture was homogenized by vortexing. After 15 min at room temperature, ureides were determined by reading in spectrophotometer (SP-220, bioespectroTM) at absorbance at 535 nm. Determination of allantoic acid was performed following almost the same process, but omitting the addition of 250 μL of NaOH 0.5 M, 20 μL of 0.33% phenylhydrazine as well as the 8-min oven-dry. The results were expressed as μmol g^{-1} DW. To quantify both allantoin and allantoic acid, a standard allantoin curve was used.

2.9. Statistical analysis

All data were submitted to Anderson-Darling normality tests; homogeneity of variance was evaluated with Leven's test and with a variance analysis (F test). Differences between treatments were compared using a Scott-Knott test at 5% probability. Analyses were conducted using R software (version 3.5.1).

3. Results

3.1. Plant dry weight

Roots, leaves + stems, and grain dry weight were affected by genotypic variation. However, the interaction between genotypes and Zn application did not affect these traits (Fig. 1; Suppl Tables 1 and 2). Roots, leaves + stems, and grains DW varied between 4.9 and 15.27 g $plant^{-1}$; 13.74 and 21.19 g $plant^{-1}$; and 1.97 and 5.67 g $plant^{-1}$ (Suppl Table 2), respectively. Two distinct groups were observed regardless of Zn application for root, leaves + stems, and grains (Suppl Table 1). For roots, genotypes 1, 2, 4, 6, 7, 8, 15, 21, and 29 had greater DW than other genotypes (Fig. 1a; Suppl Table 2). Genotypes 2, 4, 6, 11, 12, 13, 15, 17, 19, 23, 24, 25 and 26 had greater leaves + stem DW than other genotypes (Fig. 1b; Suppl Table 2). Genotypes 2, 4, 6, 10, 12, 15, 17, 23, 24, 25 e 26 had greater grain DW than other genotypes (Fig. 1c; Suppl Table 2).

3.2. Zinc concentration and partitioning in tissues

In response to Zn fertilization, grain Zn concentration varied between 47.59 and 57.11 mg kg^{-1} DW, Zn concentration in leaves + stems varied between 169.35 and 648.18 mg kg^{-1} DW, and roots Zn concentration varied between 46.73 and 432.42 mg kg^{-1} DW. Average Zn concentration in grains and leaves + stems of genotypes, in response to Zn fertilization, was 55.64 and 354.84 mg kg^{-1} DW respectively (Suppl Table 4). Root Zn concentration was affected by and interaction between Zn application and genotypes (Suppl Table 3). However, for Zn concentration in leaves + stems and grains, the interaction between

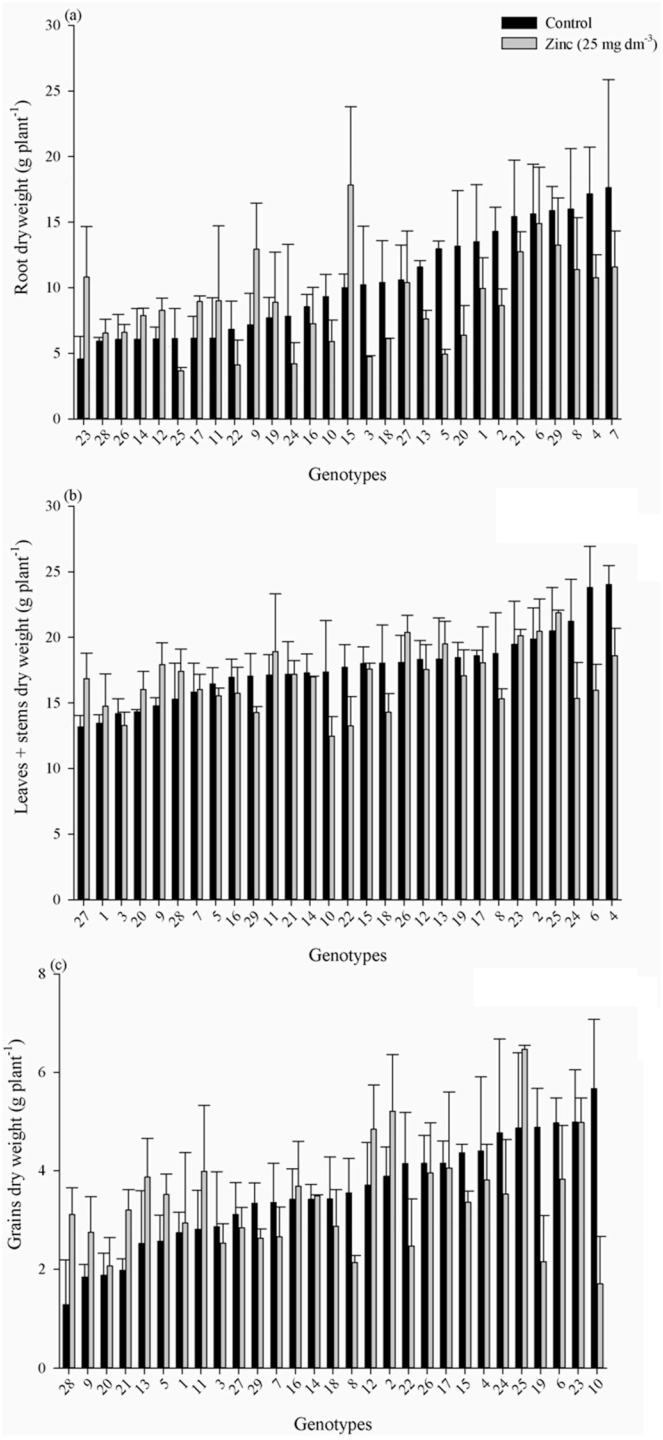

Fig. 1. Root (a), leaves + stems (b) and grain (c) dry weight (g $plant^{-1}$) of cowpea genotypes with and without application of Zn. Error bars indicates the standard error of mean (number of replicates = 3). CV (%) = 53.77 (a), 20.13 (b) and 42.49 (c). '*' Indicates difference between means of the same genotype under absence or presence of Zn application according to Scott Knott test ($p \leq 0.05$).

factors was not observed, but the isolated effect of Zn application and genotypes (Suppl Tables 3). Zinc application increased root Zn concentration in 27 out the 29 genotypes observed, only in genotypes 10 and 16, Zn fertilization did not increase Zn concentration in roots (Fig. 2c; Suppl Table 4).

Zinc concentration in grains, leaves + stems, and roots, under Zn application, could be allocated in two, three, and four groups respectively, according to the Scott Knott test. For Zn in grains, the groups with the highest Zn concentration (≥53.13 mg kg^{-1} DW) comprised genotypes 2, 3, 4, 7, 14, 16, 17, 20, 21, 28, and 29 (Fig. 2a, Suppl Table 4). And for leaves + stems Zn concentration the group with the highest Zn concentration was comprised only by genotype 4, presenting 648.18 mg kg^{-1} DW (Fig. 2b, Suppl Table 4). For Zn in roots, genotype 1 was the only one in the highest group (432.42 mg kg^{-1}; Fig. 2c; Suppl Table 4).

Zinc fertilization provided a variation in Zn partitioning to shoot between 56.9 and 95.49% and a variation in Zn partitioning to grains between 1.4 and 6.6% (Fig. 3; Suppl Table 5). The Zn partitioning to shoots and grains was affected by an interaction between Zn and genotypes (Fig. 3; Suppl Table 3). Zinc application did not increase Zn partitioning to shoot in any genotype and decrease Zn partitioning to shoot in genotypes 1, 2, 7, 8, 9, 12, 13, 15, 16, 17, 24, 26, and 29 (Fig. 3a; Suppl Tables 5). Zinc application increased Zn partitioning to grains in genotypes 2, 6, 7, 11, and 26 and decreased Zn partitioning to grains in genotypes 1, 15, 27, and 29 (Fig. 3b; Suppl Table 5).

Considering Zn partitioning to both shoots and grains, genotypes could be allocated in three groups, in response to Zn application, according to the Scott Knott test. For Zn partitioning to shoots, the group with higher partitioning (≥76,86%) comprised genotypes 2, 3, 4, 5, 10, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22, 23, 24, 25, 27 and 28 (Fig. 3a;

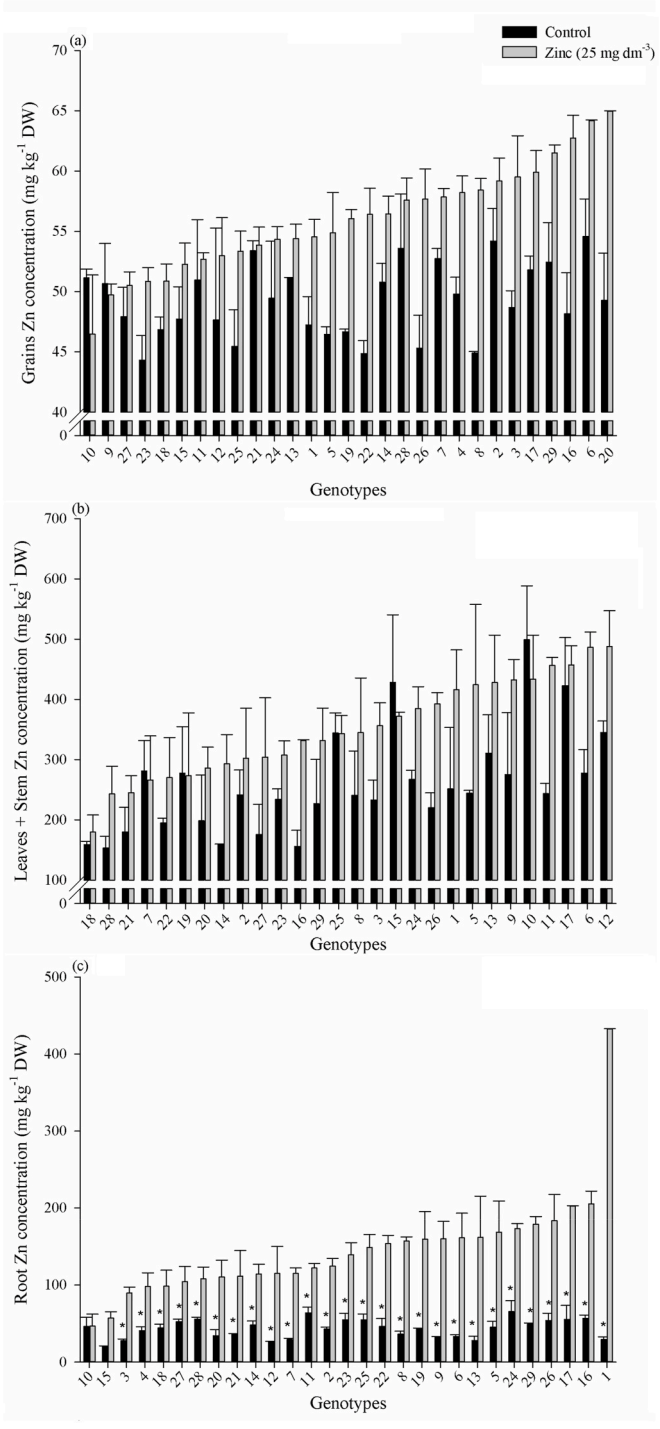

Fig. 2. Zn concentration in grains (b), leaves + stems (b) and roots (c) of cowpea genotypes with and without application of Zn. Error bars indicates the standard error of mean (number of replicates = 3). CV (%) = 8.60 (a), 44.21 (b) and 30.38 (c). '*' Indicates difference between means of the same genotype under absence or presence of Zn application according to Scott Knott test ($p \leq 0.05$).

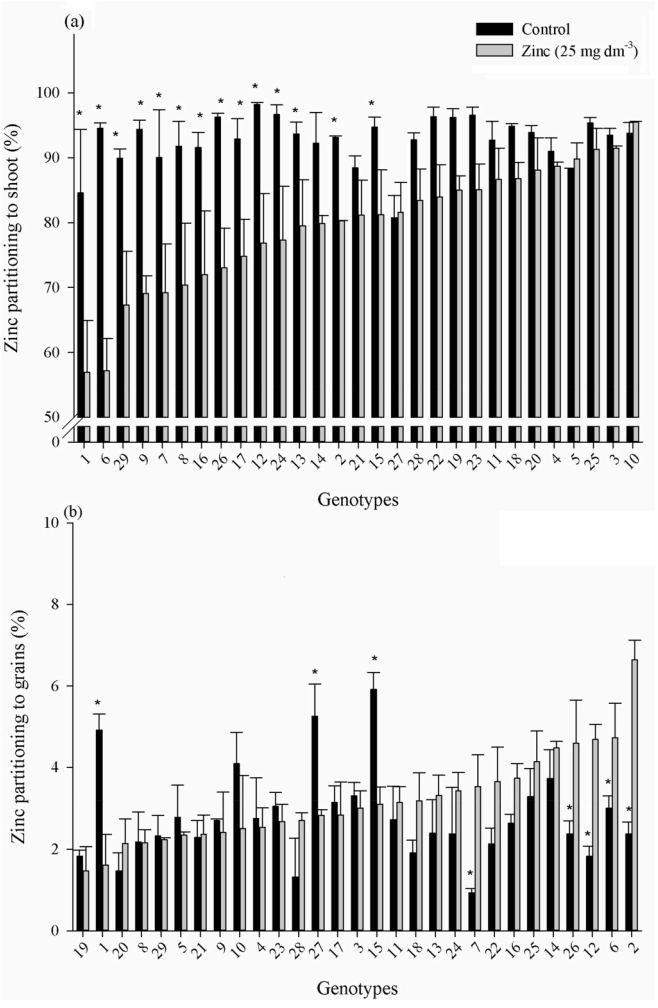

Fig. 3. Zinc partitioning to shoots (a) and grains (b) in cowpea genotypes with and without application of Zn. Error bars indicates the standard error of mean (number of replicates = 3). CV (%) = 8.98 (a) and 34.92 (b). '*' Indicates difference between means of the same genotype under absence or presence of Zn application according to Scott Knott test ($p \leq 0.05$).

Suppl Tables 5). And for Zn partitioning to grains, the group with higher partitioning, was comprised only by genotype 2 (6,64%; Fig. 3b; Suppl Tables 5).

3.3. Sucrose, total sugar, and amino acid concentration in grains

Zinc application provided a great variation in sucrose concentration between 4.77 and 9.39 mg g^{-1} DW, in total sugars between 8.09 and 14.82 mg g^{-1} DW; and in amino acids between 6.03 and 14.93 mg g^{-1} DW (Suppl Table 7). Zinc application increased sucrose concentration in grains of 12 genotypes, total sugar concentration in grains of 11 genotypes, and amino acid concentration in 15 genotypes, but decreased sucrose, total sugar, and amino acid concentration in eight, five, and three genotypes, respectively.

Sucrose, total sugars, and amino acids concentration in grains were affected by an interaction between genotype and Zn application (Fig. 4, Suppl Table 6). Zinc application increased the sucrose concentration in genotypes 1, 4, 11, 12, 15, 17, 18, 19, 22, 23, 25 and 29, and decreased the sucrose concentration in genotypes 7, 9, 10, 16, 21, 24, 26 and 28 (Fig. 4a, Suppl Table 7). Zinc application increased the total sugar concentration in genotypes 7, 8, 9, 13, 14, 19, 20, 22, 24, 27, and 29; and decreased the total sugar concentration in genotypes 1, 3, 6, 10, and 16 (Fig. 4b, Suppl Table 7). Zinc application increased amino acids concentration in genotypes 1, 10 and 14, and decreased amino acids concentration in genotypes 2, 3, 4, 5, 6, 7, 13, 17, 19, 21, 22, 24, 25, 28 and 29 (Fig. 4c, Suppl Table 7).

Sucrose and total sugar concentration could be allocated to four groups and amino acids to six groups, in response to Zn fertilization, using the Scott Knott test. For sucrose, the group with the highest sucrose concentrations (≥8.89 mg g^{-1} DW) comprised genotypes 8, 12, 15, 17, 18, 19, 22, and 29 (Fig. 4a, Suppl Table 7). For total sugar, the group with the highest concentration (≥13.06 mg g^{-1} DW) comprised genotypes 7, 14, 24, 27, and 29 (Fig. 4b, Suppl Table 7). Considering amino acid concentration, the group showing the lowest concentration (≤6.68 mg g^{-1} DW) were observed genotypes 12, 13, and 27 (Fig. 4c, Suppl Table 7).

3.4. Storage proteins

Under Zn application, albumin concentration varied between 94.53 and 126.46 mg g^{-1} DW, globulin concentration varied between 19.25 and 51.83 mg g^{-1} DW, glutelin concentration varied between 19.15 and 56.35 mg g^{-1} DW, and prolamin concentration varied between 0.52 and 1.48 mg g^{-1} DW (Suppl Tables 5 and 6).

All the storage proteins concentration were affected by an interaction between Zn application and genotypes (Fig. 5, Suppl Table 8). Zinc application increased albumin concentration in genotypes 1, 2, 9, 11, 13, 15, 17, 22, 23 and 27 and decreased albumin concentration in genotypes 3, 5, 10, 14, 16, 19, 20, 21, 24, 26, 28 and 29 (Fig. 5a, Suppl Table 5). Zinc application increased globulin concentration in genotypes 2, 3, 6, 7, 11 16, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 and 29, and decreased globulin concentration in genotypes 4, 5, 9, 10, 12, 13, 14, 15 and 17 (Fig. 5b, Suppl Table 5). Zinc application increased glutelin concentration in genotypes 3, 4, 5, 7, 10, 13, 14, 15, 16, 17, 20, 23, 24, 27 and 28, and a decrease glutelin concentration in genotype 2, 6, 8, 9, 11, 12, 18, 21, 26 and 29 (Fig. 5c, Suppl Table 10). Zinc application increased prolamin concentration in genotypes 1, 2, 3, 4, 6, 10, 11, 12, 14, 16, 28, 20, 21, 22, 25, 28 and 29, and a decreased prolamin concentration in genotypes 5, 13, 17, 19, 23, 24, 26 and 27 (Fig. 5d, Suppl Table 10).

Albumin concentration could be allocated in five groups, globulin in nine groups, glutelin in ten groups, and prolamin in six groups, in response to Zn application, using to Scott Knott test. For albumin, the group with the highest concentrations (≥118.62 mg g^{-1} DW) comprised of genotypes 1, 2, 14, 19, 20, and 21 (Fig. 5a, Suppl Table 5). For globulin, the group with the highest concentrations (≥51.81 51.81 mg g^{-1} DW) comprised by genotypes 6 and 22 (Fig. 5b, Suppl Table 5). For glutelin, the group with the highest concentrations (≥53.57 mg g^{-1} DW) comprised of genotypes 4, 13, 16, and 17 (Fig. 5c, Suppl Table 10). And for prolamin, the group with the highest concentrations (≥0.4 mg g^{-1} DW) comprised of genotypes 1, 14, 16, 20, 21, and 25 (Fig. 5d, Suppl Table 10).

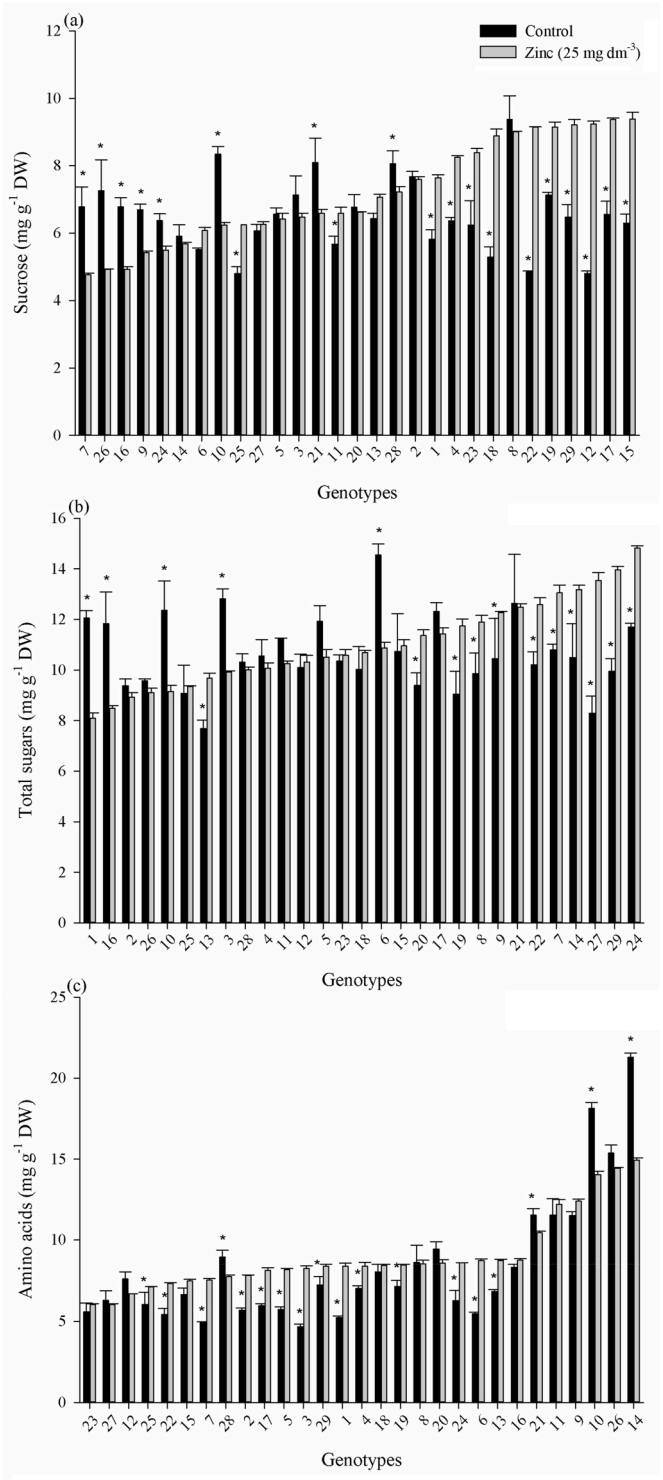

Fig. 4. Sucrose (a), total sugars (b) and amino acids (c) in grains of cowpea genotypes with and without application of Zn. Error bars indicates the standard error of mean (number of replicates = 3). CV (%) = 7.23 (a), 9.68 (b) and 6.73 (c). '*' Indicates difference between means of the same genotype under absence or presence of Zn application according to Scott Knott test ($p \leq 0.05$).

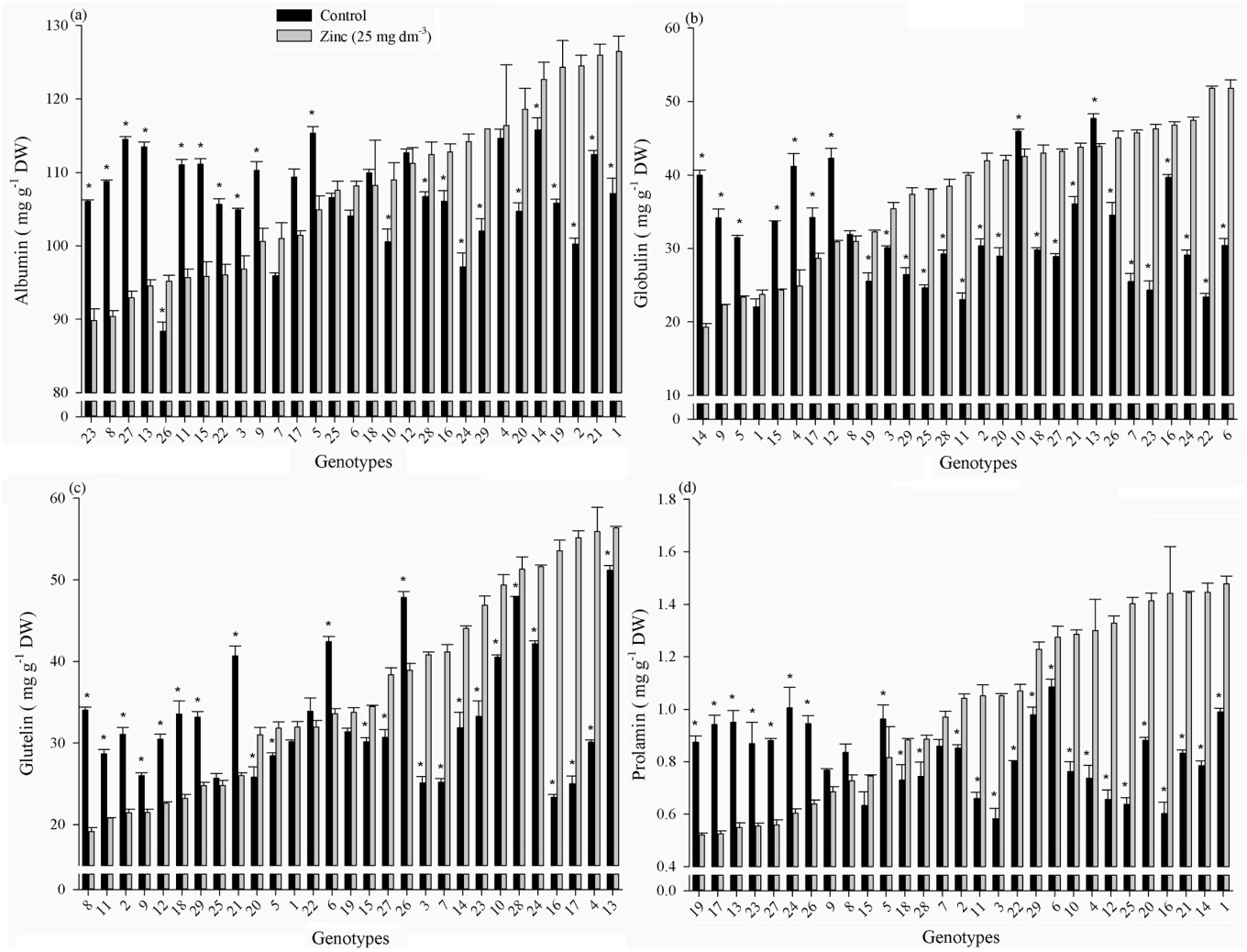

Fig. 5. Albumin (a), globulin (b), glutelin (c) and prolamin (d) concentration in grains of cowpea genotypes with and without application of Zn. Error bars indicates the standard error of mean (number of replicates = 3). CV (%) = 3.15 (a), 4.23 (b), 4.47 (c) and 8.57 (d). '*' Indicates difference between means of the same genotype under absence or presence of Zn application according to Scott Knott test ($p \leq 0.05$).

3.5. Phytic acid concentration and PA/Zn molar ratio

The application of Zn provided a variation in Phytic acid (PA) concentration between 5334 and y 10229 mg kg^{-1} DW and in PA/Zn molar ratio, a variation between 9.18 and 19.39 (Suppl Table 11).

Phytic acid (PA) concentration and PA/Zn molar ratio were affected by and interaction between genotypes and Zn application (Fig. 6; Suppl Table 8). Under Zn application, PA concentration increased in genotypes 1, 2, 4, 6, 12, 14, 17 and 19 and PA concentration decreased in 3, 7, 8, 9, 13, 18, 21, 24, 26, 27 and 29. Zinc application increased PA/Zn molar ratio in genotypes 2, 10, 12 and 17 and decrease PA/Zn molar ratio in genotypes 3, 5, 7, 8, 9, 13, 15, 16, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28 and 29.

Phytic acid concentration and PA/Zn molar ratio could be allocated, respectively, in seven and six groups, in response to Zn fertilization. The group with the lowest PA concentration (≤5519.12 mg kg^{-1} DW) comprised of genotypes 18 and 28 (Fig. 6a; Suppl Table 11). And the group for the lowest PA/Zn molar ratio (≤9.36) comprised of genotypes 20 and 28 (Fig. 6b, Suppl Table 11).

3.6. Ureides concentration in leaves + stems

In response to Zn fertilization, total ureides concentration varied between 3786.5 and 9063.53 μmol g^{-1} DW and allantoin concentration varied between 2020.87 and 7374.36 μmol g^{-1} DW (Suppl Table 12).

Total ureides and allantoin in leaves of cowpea were affected by Zn application and genotype interaction, while no results were observed for allantoic acid (Fig. 7, Suppl Table 8). Zinc application increased total ureides concentration in genotypes 2, 3,10, 12, 15, and 29; and decreased total ureides in genotype 1. Zinc application increase allantoin concentration in genotypes 2, 3, 12, 15, 23, 28, and 29 and decreased allantoin concentration in genotype 1 (Fig. 4b, Suppl Table 12).

Total ureides and allantoin concentration could be allocated, respectively, in seven and six groups due to Zn application, according to the Scott Knott test. For both total ureides and allantoin, the first group (highest concentration) was represented by genotype 21, with total ureides and allantoin concentration of 9063.53 and 7374.36 μmol g^{-1} DW, respectively (Fig. 7; Suppl Tables 12).

4. Discussion

4.1. Plant dry weight, Zn concentration, and partitioning

The soil used in this study showed a low Zn concentration (0.2 mg dm^{-3}), indicating that these 29 genotypes are Zn efficient since Zn application did not increase the dry weight of roots, leaves + stems, or grains of cowpea (Fig. 1; Suppl Tables 1 and 2). On the other hand,

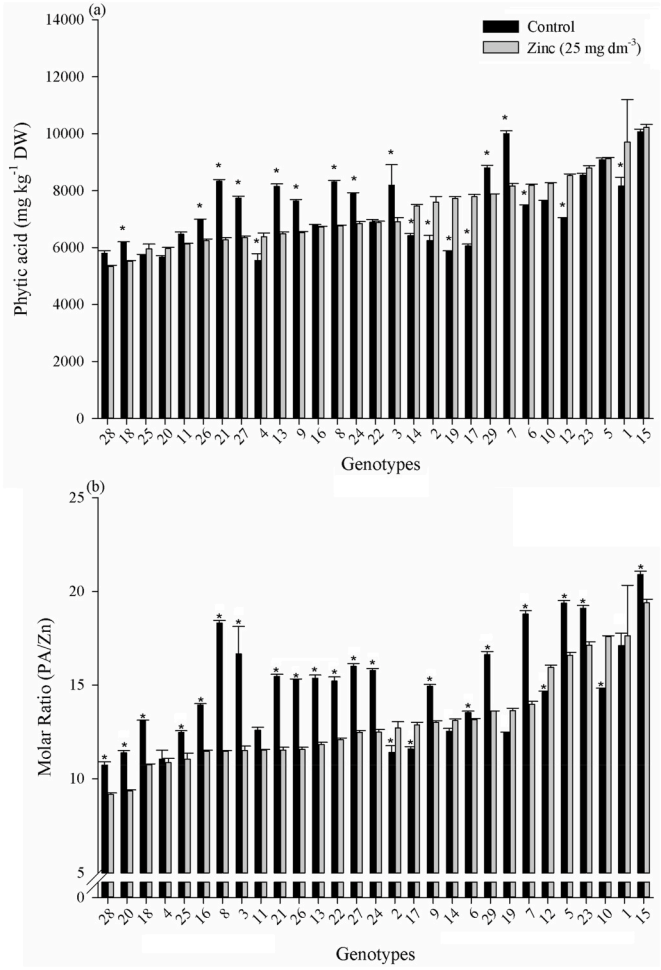

Fig. 6. Phytic acid concentration (a) and molar ratio of PA/Zn (b) in grains of cowpea genotypes with and without application of Zn. Error bars indicates the standard error of mean (number of replicates = 3). CV (%) = 5.56 (a) and 5.45 (b). '*' Indicates difference between means of the same genotype under absence or presence of Zn application according to Scott Knott test ($p \leq 0.05$).

genotypic variation was observed, and also expected, due to the high morphological variability of cowpea genotypes (Rocha et al., 2017). Genotypes 2 (BR 3-Tracuateua), 4 (BRS Cauamé), 6 (BRS Itaim), and 15 (BRS Urubuquara) were four genotypes in the group a, characterized by high root, leaf + stem, and grain DW. Possibly due to the general high capacity of cowpea genotypes to absorb Zn, no interaction between genotypes and Zn application was observed for leaves + stems and grains Zn concentration (Fig. 2; Suppl Tables 4). The critical concentration of Zn in leaves is between 15 and 20 mg kg^{-1} (Broadley et al., 2012), much lower than the concentration observed in cowpea genotypes, even in control treatments (Suppl Table 4). In previous studies, a high capacity of Zn absorption of cowpea genotypes was observed even under the low availability of Zn in soil (Oliveira et al., 2017). However, isolated effects of genotypes were observed for Zn concentration in leaves + stems and grains of cowpea in response to Zn supply (Fig. 2; Suppl Tables 4). Combining Zn, NPK, and cattle manure, Zn in grains of cowpea varied between 23 and 45 mg kg^{-1} DW (Manzeke et al., 2017), concentrations lower than those observed in this study under Zn fertilization (from 47.59 to 59.39 mg kg^{-1} DW; Suppl Tables 4). Genotype 4 (BRS cauamé) was grouped in the group "a" (high concentration) for Zn concentration in both leaves + stems and grains DW, and genotype 6 (BRS Itaim) presented the highest Zn concentration in grains DW. Is noteworthy that genotypes 16 (BRS Xiquexique), 20 (MNC04-769F-62), and 21 (MNC04-782F-108) also presented a high concentration of Zn in

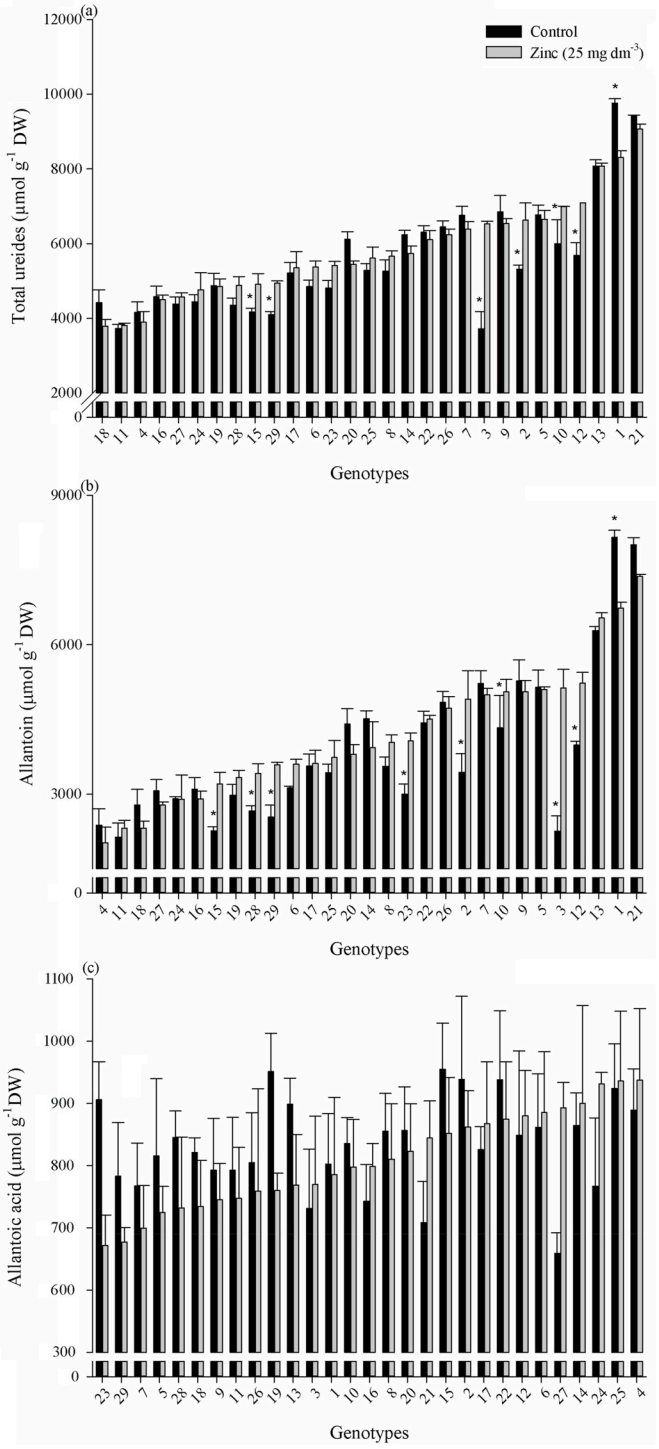

Fig. 7. Total ureides (a), allantoin (b) and allantoic acid (c) in leaves of cowpea genotypes with and without application of Zn. Error bars indicates the standard error of mean (number of replicates = 3). CV (%) = 7.37 (a), 17.56 (b) and 10.95 (c). '*' Indicates difference between means of the same genotype under absence or presence of Zn application according to Scott Knott test ($p \leq 0.05$).

grains, as previously observed by Oliveira et al. (2017).

Interestingly, the Zn application did not increase Zn partitioning to shoots but decreased partitioning in 13 genotypes (Fig. 3a; Suppl Tables 5). Most Zn accumulated in shoots of genotypes, under Zn application, was found in roots. Also, Zn partitioning to grains increased in five genotypes and decreased four (Fig. 3b; Suppl Tables 5). These results suggest that mechanism of Zn absorption by roots and translocation

to leaves + stem and grains are distinct to each genotype. Zinc efficient plants have higher Zn uptake by enhancing Zn availability in the rhizosphere leading to more efficient use of Zn within the cells (Rehman et al., 2019).

4.2. Grains quality (sucrose, total sugar, amino acid, protein, and phytic acid content)

In this study, 38% of genotypes increased in total sugar concentration, and 17% decreased in response to Zn fertilization. All genotypes that presented a decrease in total sugar concentration under Zn application (1, 3, 6, 10, and 16) also presented high total sugar concentration in the control treatment (Fig. 4b, Suppl Tables 7). For these specific genotypes, Zn application rate of 25 mg dm^{-3}, might be an impairment in sugar accumulation for these genotypes. In peanuts, total and reducing sugar concentrations increased under the treatment of soaked seeds with 300 ppm of Zn oxide as nanoparticles (Rajiv and Vanathi, 2018). In the current study, Zn application could also be inducing an increase in structural carbohydrates in some genotypes, leading to a decrease of soluble sugars accumulation in grains. As observed in guar (*Cyamopsis tetragonoloba* (L.) Zn application to soil increased cellulose and lignin in roots and decreased reducing sugars (Wadhwa and Joshi, 2016).

Zinc application increased amino acid concentration in grains of three genotypes and decreased amino acid concentration in 15 (Fig. 4c; Suppl Tables 7). All genotypes that presented a decrease in amino acids concentration, presented increases in at least one storage protein, which implies that Zn is involved in the conversion of amino acids into proteins. Genotypes 16 (BRS Xiquexique), 20 (MNC04-769F-62), and 28 (Pingo de Ouro-2) presented an increase in all storage proteins analyzed. Zinc application increased the activity of nitrate reductase and glutamine synthetase in wheat, leading to an increase in globulin, albumin, and glutelin in grains (Liu et al., 2015). Thus, Zn could be increasing grain protein concentration in cowpea due to its effect on the activities of nitrogen assimilation enzyme pathways. In previous studies of evaluation of nutritional quality in grains of 11 genotypes of cowpea, a positive correlation between Zn and protein concentration was observed (Moura et al., 2012).

In this study, Zn application decreased PA concentration in 11 genotypes and increased it in seven (Fig. 6a, Suppl Tables 11). Zinc might decrease phosphorus absorption by roots (Bharti et al., 2013), which might decrease PA concentration. However, Zn application did not decrease phosphorus concentration in cowpea tissues (Suppl Tables 3 and 13). Molar ratios of PA/Zn higher than 15 reduce Zn availability by humans (Gargari et al., 2007). Under Zn fertilization, only genotypes 5 (BRS Guariba), 10 (BRS Novaera), 12 (BRS Potengi), 15 (BRS Urubuquara) and 23 (MNC04-792F-146) presented PA/Zn molar ratio higher than 15. The PA/Zn molar ratio decreased in 19 genotypes response to Zn fertilization, indicating a general increase in Zn availability of cowpea grains. Studying three genotypes of common bean, Figueiredo et al. (2017), observed that Zn application decreased PA concentration in grains. While a general decrease in PA/Zn molar ratio was observed, due to wide variation in genotypes, some lines presented the inverse behavior to that expected and observed in most genotypes. In four genotypes: 2 (BR 3-Tracuateua), 10 (BRS Novaera), 12 (BRS Potengi), and 17 (California CB-5), Zn application increased PA/Zn molar ratio. The enhancement of Zn bioavailability provided by the decrease in PA/Zn molar ratio in this group of genotypes is valuable information for breeding programs aiming to reduce undernutrition originating from PA concentrations.

4.3. Ureides

Ureides are translocated from root to shoot through the xylem and converted into NH_4^+ and amino acids in the cytosol of leaf tissue (Baral et al., 2016). Allantoin and total ureides concentration showed genotype-specific variation in response to Zn fertilization. Similar results between total ureides and allantoin is due to allantoic acid concentrations being very low (total ureides are represented by allantoin + allantoic acid). Ureides concentration was affected in few genotypes in this study, as observed in three genotypes of soybean under Zn application of 0, 5, 10, 20, and 40 mg kg^{-1}, in which, Zn application did not affect ureides concentration (Moreira et al., 2016). Although only a few genotypes presented increases in ureides concentration under Zn application, it might be a start for future studies, since is possible that Zn plays a role in nitrogen biological fixation (Stowhas et al., 2018). In future studies regarding Zn and nitrogen fixation interaction, genotypes 2 (BR 3-Tracuateua), 3 (BRS Aracê), 12 (BRS Potengi), 15 (BRS Urubuquara), and 29 (Pretinho) might be good candidate genotypes. Mostly, genotypes 2, 3, and 29 also presented a decrease in amino acid concentration under Zn application, indicating not only an effect of Zn in nitrogen fixation but also its incorporation into proteins.

5. Conclusion

Cowpea presented wide genotypic variation in traits measured here, such as total sugar concentration, amino acids, PA concentration, Zn availability across the 29 genotypes studied. The information compiled in this study showed that Zn partitioning to shoots and grains might be a very useful trait to identify genotypes showing high potential to accumulate Zn in cowpea grains.

Depending on each genotype, Zn fertilization has the potential to increase Zn and storage protein concentration, as well as decrease PA and the PA/Zn molar ratio in cowpea grains. Zinc fertilization increased ureides concentration in some cowpea genotypes. This information could lead to further studies for a better understanding of Zn physiological role on biological nitrogen fixation in cowpea plants.

This study provides important information for future breeding programs aiming to enhance grain nutritional quality by decreasing PA and increasing Zn concentration in cowpea grains.

Contributions

VMS wrote the manuscript and run the analysis in the laboratory, AJN and NACM carried out the experiment from sowing to crop and helped VMS in writing and lab analysis. MMR provided the genetic material used in the experiment, revised the manuscript, and contributed to planning the experiment. LW performed lab analysis, data compilation, and interpretation with VMS. SDY, MRB, and PJW co-supervised the experiment and revised the manuscript. ARR was the major supervisor of the experiment, providing financial support for the project, planning the analysis, and advising, mainly, VMS, AJN, and NACM.

Credit authorship contribution statement

Vinícius Martins Silva: Conceptualization, Investigation, Methodology, Validation, Writing – original draft. **Ana Júlia Nardeli:** Investigation, Methodology, Validation. **Nandhara Angélica de Carvalho Mendes:** Investigation, Methodology, Validation. **Maurisrael de Moura Rocha:** Investigation, Methodology, Validation. **Lolita Wilson:** Investigation, Methodology, Validation. **Scott D. Young, Martin R. Broadley and Philip J. White:** Investigation, Methodology, Validation, review & editing. **André Rodrigues dos Reis:** Conceptualization, Validation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors wish to thank the São Paulo State Research Support Foundation (FAPESP) for supporting the first author's research with financial resources provided by process number 18/18936-6. ARR also thanks to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the research fellowship (Grant number 309380/2017-0). Additional thanks go to Thomas D Alcock at the Future Food Beacon, University of Nottingham, for early feedback on this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2021.02.020.

References

Abichequer, A.D., Bohnen, H., 1998. Eficiência de absorção, translocação e utilização de fósforo por variedades de trigo. Rev. Bras. Ciência do Solo 22, 21–25. https://doi.org/10.1590/S0100-06831998000100003.

Baral, B., Silva, J.A.T., Izaguirre-Mayoral, M.L., 2016. Early signaling, synthesis, transport and metabolism of ureides. J. Plant Physiol. 193, 97–109. https://doi.org/10.1016/j.jplph.2016.01.013.

Bharti, K., Pandey, N., Shankhdhar, D., Srivastava, P.C., Shankhdhar, S.C., 2013. Improving nutritional quality of wheat through soil and foliar zinc application. Plant Soil Environ. 59, 348–352. https://doi.org/10.17221/158/2013-PSE.

Bielesk, R.L., Turner, N.A., 1966. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. Anal. Biochem. 17, 278–293. https://doi.org/10.1016/0003-2697(66)90206-5.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–259. https://doi.org/10.1016/0003-2697(76)90527-3.

Broadley, M.R., Brown, P., Cakmak, I., Rengel, Z., Zhao, F., 2012. Function of nutrients: micronutrients. In: MARSCHNER, P. (Ed.), Marschner's Mineral Nutrition of Higher Plants, third ed. Academic Press, San Diego, pp. 191–248. https://doi.org/10.1016/B978-0-12-384905-2.00007-8.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356. https://doi.org/10.1021/ac60111a017.

Gargari, B.P., Mahboob, S., Razavieh, S.V., 2007. Content of phytic acid and its mole ratio to zinc in flour and breads consumed in Tabriz, Iran. Food Chem. 100, 1115–1119. https://doi.org/10.1016/j.foodchem.2005.11.018.

Grewal, S.K., Sharma, K.P., Bharadwaj, R.D., Hedge, V., Tripathi, S., Singh, S., Jain, P.K., Agrawal, P.K., Mondaf, B., 2020. Understanding genotypic variation and identification of promising genotypes for iron and zinc content in chickpea (*Cicer arietinum* L.). J. Food Compos. Anal. 88, 103458. https://doi.org/10.1016/j.jfca.2020.103458.

Gödecke, T., Stein, A.J., Qaim, M., 2019. The global burden of chronic and hidden hunger: trends and determinants. Global Food Security 17, 21–29. https://doi.org/10.1016/j.gfs.2018.03.004.

Haider, M.U., Hussain, M., Farooq, M., Nawaz, A., 2020. Zinc nutrition for improving the productivity and grain biofortification of mungbean. J. Soil Sci. Plant Nutr. https://doi.org/10.1007/s42729-020-00215-z.

Joy, E.J.M., Ander, L., Young, S.D., Black, C.R., Watts, M.J., Chilimba, A.D.C., Chilima, B., Siyame, E.W., Kalimbira, A.A., Hurst, R., Fairweather-Tait, S.J., Stein, A. J., Gibsion, R.S., White, P.J., Broadley, M.R., 2014. Dietary mineral supplies in Africa. Physiol. Plantarum 151, 208–229. https://doi.org/10.1111/ppl.12144.

Kumar, S., Palve, A., Joshi, C., Srivastava, R.K., Ruchsar, 2017. Crop biofortification for iron (Fe), zinc (Zn) and vitamin A with transgenic approaches. Helyon 5, e01914. https://doi.org/10.1016/j.heliyon.2019.e01914.

Kumar, S., Pandey, G., 2020. Biofortification of pulses and legumes to enhance nutrition. Heliyon 6, e03682. https://doi.org/10.1016/j.heliyon.2020.e03682.

Kumssa, D.B., Joy, E.J.M., Ander, E.L., Watts, M.J., Young, S.D., Walker, S., Broadley, M. R., 2015. Dietary calcium and zinc deficiency risks are decreasing but remain prevalent. Sci. Rep. 5, 10974. https://doi.org/10.1038/srep10974.

Liu, H.E., Wang, Q.Y., Rengel, Z., Zhao, P., 2015. Zinc fertilization alters flour protein composition of winter wheat genotypes varying in gluten content. Plant Soil Environ. 61, 195–200. https://doi.org/10.17221/817/2014-PSE.

Lividini, K., Fiedler, J.L., De Moura, F.F., Moursi, M., Zeller, M., 2018. Biofortification: a review of ex-ante models. Global Food Security 17, 186–195. https://doi.org/10.1016/j.gfs.2017.11.001.

Manzeke, M.G., Mtambenengwe, F., Nezomba, H., Mapfumo, P., 2014. Zinc fertilization influence on maize productivity and grain nutritional quality under integrated soil fertility management in Zimbabwe. Field Crop. Res. 166, 128–136. https://doi.org/10.1016/j.fcr.2014.05.019.

Manzeke, M.G., Mtambenengwe, F., Nezomba, H., Watts, M.J., Broadley, M.R., Mapfumo, P., 2017. Zinc fertilization increases productivity and grain nutritional quality of cowpea (*Vigna unguiculata* [L.] Walp.) under integrated soil fertility management. Field Crop. Res. 213, 231–244. https://doi.org/10.1016/j.fcr.2017.08.010.

Manzeke, M.G., Mtambanengwe, F., Watts, M.J., Broadley, M.R., Murray, L.R., Mapfumo, P., 2020. Nitrogen effect on zinc biofortification of maize and cowpea in Zimbabwean smallholder farms. Agron. J. https://doi.org/10.1002/agj2.20175.

Masuda, H., Aung, M.S., Kobayashi, T., Nishizawa, N.K., 2020. Iron biofortification: the gateway to overcoming hidden hunger. In: COSTA DE OLIVEIRA, A., PEGORARO, C., EBELING VIANA, V. (Eds.), The Future of Rice Demand: Quality beyond Productivity. Springer, Cham, pp. 149–177. https://doi.org/10.1007/978-3-030-37510-2_7.

Moreira, A., Moraes, L.A.C., Furlan, T., Cerezini, P., Bruno, I.P., 2016. Interaction of glyphosate with zinc for the yield, photosynthesis, soil fertility and nutritional status of soybean. Commun. Soil Sci. Plant Anal. 47, 706–716. https://doi.org/10.1080/00103624.2016.1146747.

Moura, J.O., Rocha, M.M., Gomes, R.L.F., Freire Filho, F.R., Damasceno, K.J., Ribeiro, V. Q., 2012. Path analysis of iron and zinc contents and others traits in cowpea. Crop Breeding and Applied Biotechnology 12, 245–252. https://doi.org/10.1590/S1984-70332012000400003.

Oliveira, D.S.V., Franco, L.J.D., Menezes-Júnior, J.A.N., Damasceno-Silva, K.J., Rocha, M.M., Neves, A.C., Sousa, F.M., 2017. Adaptability and stability of the zinc density in cowpea genotypes through GGE-Biplot method. Ciência Agronômica 48, 783–791. https://doi.org/10.5935/1806-6690.20170091.

Philipo, M., Ndakidemi, P.A., Mbega, E.R., 2020. Environmental and genotypes influence on seed iron and zinc levels of landraces and improved varieties of common bean (*Phaseolus vulgaris* L.) in Tanzania. Ecological Genetics and Genomics 15, 100056. https://doi.org/10.1016/j.egg.2020.100056.

Quaggio, J.A., Raij, B. van, 1997. Correção da acidez do solo. In: RAIJ, B.V.A.N., CANTARELLA, H., QUAGGIO, J.A., FURLANI, A.M.C. (Eds.), Recomendações de adubação e calagem para o Estado de São Paulo. Instituto Agronômico de Campinas, Campinas, pp. 13–18, 1997b, (IAC. Boletim Técnico, 100).

Raij, B.van, Andrade, J.C., Cantarella, H., Quaggio, J.A., 1997. Análise Química Para Avaliação da Fertilidade de Solos Tropicais. Instituto Agronômico de Campinas, Campinas, p. 285.

Rajiv, P., Vanathi, P., 2018. Effect of *Parthenium* based vermicompost and zinc oxide nanoparticles on growth and yield of *Arachis hypogaea* L. in zinc deficient soil. Biocatalysis and Agricultural Biotechnology 13, 251–257. https://doi.org/10.1016/j.bcab.2018.01.006.

Rehman, A., Farooq, M., Asif, M., Ozturk, L., 2019. Supra-optimal growth temperature exacerbates adverse effects of low Zn supply in wheat. J. Plant Nutr. Soil Sci. 182, 656–666. https://doi.org/10.1002/jpln.201800654.

Rocha, M.M., Damasceno-Silva, K.J., Menezes-Júnior, J.A.N., 2017. Cultivares. In: Do Vale, J.C., Bertini, C., Borém, A. (Eds.), Feijão-caupi: do plantio à colheita. Editora UFV, Viçosa, pp. 113–142.

Stowhas, T., Verdejo, J., Yáñez, C., Celis-Diez, J.L., Martínez, C.E., Neaman, A., 2018. Zinc alleviates copper toxicity to symbiotic nitrogen fixation in agricultural soil affected by copper mining in central Chile. Chemosphere 209, 690–693. https://doi.org/10.1016/j.chemosphere.2018.06.166.

Silva, V.M., Boleta, E.H.M., Martins, J.T., Dos Santos, F.L.M., Silva, A.C.R., Alcock, T.D., Wilson, L., De, Sá M.E., Young, S.D., Broadley, M.R., White, P.J., Reis, A.R., 2019. Agronomic biofortification of cowpea with selenium: effects of selenate and selenite applications on selenium and phytate concentrations in seeds. J. Sci. Food Agric. 99, 5969–5983. https://doi.org/10.1002/jsfa.9872.

Teka, T.A., Retta, N., Bultosa, G., Admassu, H., Astatkie, T., 2020. Protein fractions, in vitro protein digestibility and amino acid composition of select cowpea varieties grown in Ethiopia. Food Bioscience. https://doi.org/10.1016/j.fbio.2020.100634 (in press).

Thomas, C.L., Alcock, T.D., Graham, N.S., Hayden, R., Matterson, S., Wilson, L., Young, S.D., Dupuy, L.X., White, P.J., Hammond, J.P., Danku, J.M.C., Salt, D.E., Sweeney, A., Bancroft, I., Broadley, M.R., 2016. Root morphology and seed and leaf ionomic traits in a Brassica napus L. diversity panel show wide phenotypic variation and are characteristic of crop habit. BMC Plant Biol. 16, 214. https://doi.org/10.1186/s12870-016-0902-5.

Van Handel, E., 1968. Direct microdetermination of sucrose. Anal. Biochem. 22, 280–283. https://doi.org/10.1016/0003-2697(68)90317-5.

Vogels, G.D., Van Der Drift, C., 1970. Differential analyses of glyoxylate derivates. Anal. Biochem. 33, 143–157. https://doi.org/10.1016/0003-2697(70)90448-3.

Wadhwa, N., Joshi, U.N., 2016. Zinc supplementation induces resistance against root-rot in guar [*Cyamopsis tetragonoloba* (L.) Taub] seedlings. Australas. Plant Pathol. 45, 465–471. https://doi.org/10.1007/s13313-016-0439-y.

Yemm, E.W., Cocking, E.C., Ricketts, R.E., 1954. Estimation of amino acids by ninhydrin. Analyst 58, 209–214.