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Fertilization/ Original Article

Agronomic biofortification of common bean grain with zinc

Abstract – The objective of this work was to evaluate the effects of the split and combined application of foliar zinc (ZnF) + soil Zn (ZnS) on Zn concentration in the grains (ZnCG) of common bean (*Phaseolus vulgaris*). Two experiments were carried out in a greenhouse during two seasons. In the first experiment, two common bean cultivars received six ZnF rates (0, 120, 240, 480, 720, or 1,200 g ha⁻¹), with split applications at the R5, R7, and R8, or R7 and R8, or R8 plant stages. In the second experiment, one cultivar received three ZnS rates (0, 5, or 10 kg ha⁻¹) combined with ZnF rates (0, 1, 1.5, 3, 6, or 10 kg ha⁻¹). In the first experiment, with the ZnF increment, ZnCG increased linearly from 15.3 to 20.7 mg kg⁻¹. ZnF splitting did not affect ZnCG. In the second experiment, ZnF without ZnS did not affect ZnCG. ZnS doubled ZnCG in comparison with the treatment without ZnS application. At 5 kg ha⁻¹ ZnS, the highest ZnCG (67.5 mg kg⁻¹) was obtained with 7.8 kg ha⁻¹ ZnF. At 10 kg ha⁻¹, the highest ZnCG (66 mg kg⁻¹) was obtained with 4.9 kg ha⁻¹ ZnF. ZnF splitting is not advantageous over a single application, and the combination of ZnF and ZnS increases ZnCG in common bean, in greenhouse conditions.

Index terms: *Phaseolus vulgaris*, concentration of Zn in grains, food quality, zinc fertilization.

Biofortificação agrônômica de grãos de feijão com zinco

Resumo – O objetivo deste trabalho foi avaliar os efeitos da aplicação parcelada e combinada de zinco foliar (ZnF) + Zn no solo (ZnS) sobre o teor de Zn dos grãos (TZnG) do feijão-comum (*Phaseolus vulgaris*). Dois experimentos foram realizados em casa de vegetação durante dois períodos. No primeiro experimento, duas cultivares de feijão-comum receberam seis doses de ZnF (0, 120, 240, 480, 720, ou 1.200 g ha⁻¹), com aplicações parceladas nos estágios da planta R5, R7 e R8, ou R7 e R8, ou R8. No segundo experimento, uma cultivar recebeu três doses de ZnS (0, 5 ou 10 kg ha⁻¹) combinadas com doses de ZnF (0, 1, 1,5, 3, 6 ou 10 kg ha⁻¹). No primeiro experimento, com o incremento do ZnF, o TZnG aumentou linearmente de 15,3 para 20,7 mg kg⁻¹. O parcelamento do ZnF não afetou o TZnG. No segundo experimento, o ZnF sem o ZnS não afetou o TZnG. O ZnS dobrou o TZnG em comparação ao tratamento sem aplicação de ZnS. Com 5 kg ha⁻¹ de ZnS, o mais alto TZnG (67,5 mg kg⁻¹) foi obtido com 7,8 kg ha⁻¹ de ZnF. Com 10 kg ha⁻¹, o mais alto TZnG (66,0 mg kg⁻¹) foi obtido com 4,9 kg ha⁻¹ de ZnF. O parcelamento do ZnF não é vantajoso em relação à aplicação única, e a combinação ZnF e ZnS aumenta o TZnG do feijão-comum em casa de vegetação.

Termos para indexação: *Phaseolus vulgaris*, teor de Zn nos grãos, qualidade alimentar, adubação com Zn.

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Introduction

Biofortification is a mean to increase the Zn content (and other minerals and vitamins) in the edible parts of crop plants. Agronomic biofortification with Zn fertilization of crop plants is a rapid solution to enrich food with this micronutrient. Biofortification techniques aim at increasing Zn concentration in edible plant parts, besides increasing the proportion of bioavailable Zn. On average, the Zn bioavailability is 25% of the Zn ingested (Clemens, 2014).

Common bean (*Phaseolus vulgaris* L.) is the most cultivated grain legume in the world, and it is the main staple food for lower-income populations of many countries in America and Africa (Blair, 2013). The average Zn concentration in common bean grains is 35 mg kg⁻¹, which is among the highest ones for crop plants (Beebe et al., 2000). Therefore, common bean grains has been a target for biofortification, aiming at increasing the Zn concentration to 56 mg kg⁻¹ (White & Broadley, 2011). Although common bean is considered a crop with low-Zn use efficiency (Moreira et al., 2018), grain-Zn concentration over 56 mg kg⁻¹ was obtained only with foliar Zn application in Brazil (Ram et al., 2016).

Biofortification studies worldwide concentrate, in general, on cereals as rice and wheat. Zn foliar (ZnF) application has increased the Zn concentration in grains (ZnCG) of cereals, especially wheat, whereas Zn applied to soil (ZnS) at sowing generally had little effect on ZnCG of cereals under field conditions. The combination of ZnF and ZnS has provided small increase of ZnCG in cereals, in comparison to ZnF alone (Cakmak & Kutman, 2018).

Few studies have evaluated the effect of the combined ZnS and ZnF on ZnCG of grain legumes. In raw grains of pea, ZnS (0, 4, or 8 mg ZnSO₄ 7H₂O kg⁻¹ soil) increased ZnCG from 34 to 70 mg kg⁻¹, but ZnF alone increased more efficiently the ZnCG (Poblaciones & Rengel, 2016). Thus, results with ZnS in pea are more encouraging than those for cereals aiming to increase ZnCG. A study conducted with common bean in five sites in São Paulo state, Brazil, indicates that ZnF increases ZnCG between 4 and 30% (Ram et al., 2016). However, the effects of ZnS + ZnF on ZnCG have not been studied in common bean.

Studies with wheat (Cakmak et al., 2010; Ajiboye et al., 2015) and rice (Boonchuay et al., 2013) were carried out to test ZnF at different stages of this crop

development (or combinations of stages) with a fixed rate of Zn (4 to 5 kg ha⁻¹ of ZnSO₄·7H₂O). Results indicated that ZnF should be applied after flowering for rice, and during grain-filling for wheat. We did not find any study examining the effect of splitting Zn fertilizers applied on foliage into two or three applications, at different growth stages of common bean (or other crop) on ZnCG.

The objective of this work was to evaluate the effects of splitting Zn fertilizer into two or three applications on grain Zn concentration of common bean, and the effectiveness of the combined Zn fertilization (soil + foliar) on grain Zn concentration.

Materials and Methods

Two experiments were carried out in a greenhouse in the municipality of Viçosa (20°45'S, 42°54'W, at 650 m altitude), in the state of Minas Gerais, Brazil. The experimental units consisted of 5 dm³ plastic pots, without holes in the bottom, filled with 3 dm³ of sieved (2 mm) air-dried Latossolo Vermelho-Amarelo (Santos et al., 2013), i.e., Oxisol. The original characteristics of the soil were: 5.3 pH in water (1:2.5); 1.7 dag kg⁻¹ organic matter (Walkley-Black); 0.9 mg dm⁻³ available P; 7.0 mg dm⁻³ available K (Mehlich-1); 0.65, 0.02, 0.70, and 4.10 cmol_c dm⁻³ exchangeable Ca, Mg, Al, and H+Al (0.5 mol L⁻¹ calcium acetate extractor), respectively; 13.1 mg L⁻¹ remaining P (Mehlich-1 extractor); and 5.2, 87.4, and 1.02 mg dm⁻³ of Mn, Fe, and Zn (Mehlich-1), respectively. The Zn availability in the soil is considered intermediate (Ribeiro et al., 1999).

Based on the soil analysis, acidity was corrected by Al neutralization (Ribeiro et al., 1999). CaCO₃ and MgCO₃ were mixed with soil to a 4:1 ratio. Then, distilled water was added to the soil, and shaken in a plastic bag until the soil reached the moisture at field capacity. The soil was then incubated for 20 days. After that, 100 mL of macro- and micronutrient solution were mixed with the soil. This solution contained the following nutrients (in mg kg⁻¹ of soil): 100 N, 300 P, 150 K, 40 S, 0.81 B, 1.33 Cu, 1.55 Fe, 3.66 Mn, 0.15 Mo, and 4.00 Zn (Novais et al., 1991). For Zn, however, the amount recommended by Novais et al. (1991) was reduced from 4.00 to 1.50 mg kg⁻¹ of soil because of the addition of Zn that would be applied to foliage, and the intermediate Zn available in the soil. In the

second experiment, the Zn source of this solution was eliminated.

Five seeds were sown per pot and, after emergence, the seedlings were thinned to two plants per pot. At 30 days after planting, a solution of ammonium nitrate was applied to the soil at 64 kg ha⁻¹ of N. This application was repeated at 45 days after planting. The soil was watered daily with distilled water to keep it moist, but not soaked.

The first experiment, from February to May 2014, lasted 137 days. The temperature inside the greenhouse ranged from 15 to 38°C. The treatments were arranged in a (2x5x3)+2 factorial design, with: the common bean cultivar BRSMG Madrepérola or Carioca 1030; ZnF rates at 120, 240, 480, 720, or 1200 g ha⁻¹; split applications to plants at the growth stages R5, R7, and R8, or R7 and R8, or at R8 only, plus zero rate for each cultivar. Both cultivars belong to “carioca” type and have indeterminate type III growth habit. The R5 growth stage corresponds to the pre-flowering, R7 to the pod formation, and R8 to the pod filling. The split Zn rates were divided into equal sub-rates. Treatments were replicated four times, in a randomized complete block design.

The Zn source was ZnSO₄·7H₂O; the Zn solution was applied to the foliage using a hand-held sprayer with flat fan nozzle. Before the application, the amount of water required to wet the two plants was estimated for each pot with minimum run off. Thus, the solution volume increased as plant growth progressed: 20 mL at R5, 40 mL at R7, and 50 mL at R8.

Grain production per pot and ZnCG were evaluated. Grain production was estimated with grains at 12% moisture content (wet basis). For the ZnCG analysis, 15 grains of each replicate were dried in an air-circulation oven at 65°C until a constant mass was achieved and, then, milled in a Wiley mill with a 20-mesh sieving screen. Milled grains were weighted (0.5 g) and digested using a nitro-perchloric solution. Grain-Zn concentrations were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Malavolta et al., 1997).

Based on the results of the first experiment, the second one was planned. The second experiment lasted 126 days, from August to November 2014. Temperature in the greenhouse ranged from 13 to 40°C. Treatments were arranged in a 3x6 factorial design, which were rates of Zn at 0, 5, or 10 kg ha⁻¹ applied to the soil; and

ZnF at 0, 1, 1.5, 3, 6 or 10 kg ha⁻¹. ZnF rates were much higher than in the first experiment, when their effect on ZnCG was linear. As the ZnCG of the two cultivars used in the first experiment was similar, we used for this experiment the most recently released cultivar BRSMG Madrepérola. Treatments were replicated four times, in a randomized complete block design.

For Zn applied to soil (ZnS), we mixed ZnSO₄ with the total soil volume of each pot on the planting day, according to Poblaciones & Rengel (2016). For ZnF, 20 mL of ZnSO₄ solution was enough to wet the two plants, at the R8 growth stage, of each pot with minimum run off, since plants in this experiment grew less than those of the first one. The R8 growth stage was chosen for ZnF application because the splitting had no effect on ZnCG, and the maximum Zn uptake takes place in this growth stage of common bean (Moreira et al., 2018). Grain production per plant (with 12% water) and grain concentrations of P, K, Ca, Mg, S, Fe, and Zn were determined in the second experiment. For this, grains were dried, ground, and digested using a nitro-perchloric solution (Malavolta et al., 1997). The nutrient content was determined by ICP-OES.

To localize Zn in the grain tissues, one grain (harvested in the second experiment) was used with each Zn concentration (22, 61, and 89 mg kg⁻¹). The cotyledons of each grain were separated from each other using a razor blade, and the cotyledon with the embryo axis attached was glued to a glass plate. Cotyledons on the plates were examined using an energy-dispersive X-ray spectrometry (EDX) to generate images.

Factorial experiments were analyzed by three-way (first experiment) or two-way (second experiment) analysis of variation, using the statistic program SAEG 9.1. The effects of Zn rates were evaluated by regression analysis. In the first experiment, we used two degrees of freedom to compare the zero rate with the other Zn rates. In the second experiment, when ZnS x ZnF interaction was significant, the effects of the ZnF rates were tested within each ZnS rate.

Results and Discussion

In the first experiment, cultivar, Zn rate, splitting, and interactions had no significant effects on grain production per pot. The soil analysis indicated intermediate Zn availability in the soil (Ribeiro et

al., 1999), which together with the Zn fertilization at planting (3 kg ha⁻¹) are the probable reasons for this lack of significant effects of Zn rates on grain yield.

Cultivars ($p = 0.67$), splitting ($p = 0.55$), and cultivar x Zn rate ($p = 0.33$), cultivar x splitting ($p = 0.67$), and cultivar x Zn rate x splitting ($p = 0.17$) interactions did not affect ZnCG. In wheat, however, the highest ZnCG were obtained when Zn was applied two (Ajiboye et al., 2015), or four (Cakmak et al., 2010) times. However, in these cases, the Zn rate was not split, that is, 4 kg of ZnSO₄·7H₂O were applied at each growth stage. Further studies of agronomic biofortification of common bean with Zn in the field are necessary to complement and validate our results in greenhouse.

The contrast zero rate vs. other Zn rates and the effects of ZnF rates were significant ($p < 0.001$) on ZnCG. On average, at zero rate, ZnCG was 15.3 mg kg⁻¹ (Figure 1). The increase of ZnF rates from 120 to 1,200 g ha⁻¹ led to a linear increase in ZnCG to up 20.7 mg kg⁻¹. Comparing with the zero rate, the application of 1,200 g ha⁻¹ of Zn increased ZnCG by 35%. We did not use Zn rates higher than 1,200 g ha⁻¹ to prevent a possible leaf scorch that would be caused by the Zn solution because the highest rate of ZnF tested for correction of Zn deficiency in common bean is 800 g ha⁻¹ (Teixeira et al., 2008). The highest ZnCG achieved in this greenhouse experiment was below the range of 53.2 to 81.2 mg kg⁻¹ of Zn obtained by Ram et al. (2016) in field experiments in Brazil, probably indicating that Zn available to plants in the present

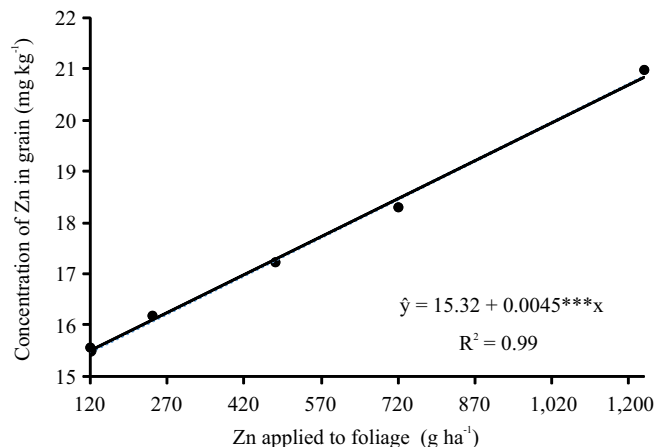


Figure 1. Effects of zinc (Zn) application to the foliage of common bean (*Phaseolus vulgaris*) on Zn concentration in grains, in a greenhouse experiment.

study was low. Our results associated with those of Ram et al. (2016) suggest that ZnF in common bean could increase ZnCG by 4 to 35%. In cereals, ZnF increases ZnCG more in wheat (40 to 420%) than in rice (10 to 60%) and maize (40%) (Shahzad et al., 2014).

In the second experiment, ZnF ($p = 0.30$) and ZnS x ZnF interaction ($p = 0.90$) did not affect grain production per pot. However, ZnS increased the grain production ($p < 0.001$) from 2.1 g (without Zn) to 13.8 g (5 kg ha⁻¹ Zn) and 15.1 g (10 kg ha⁻¹ Zn). Bean plants growing on soil not fertilized with Zn exhibited symptoms of Zn deficiency similar to those reported by Kabir et al. (2014).

ZnS x ZnF interaction had a marginal significance ($p = 0.058$) on ZnCG. Decomposition of this interaction showed that when ZnS was not used, the effect of ZnF on ZnCG was nonsignificant (Figure 2). It is possible that the leaf absorption of ZnF, and Zn translocation into pods and deposited into grains might be impaired by biochemical and histological changes in the plants caused by Zn deficiency (Kabir et al., 2014). ZnF rates affected ZnCG within 5 and 10 kg ha⁻¹ ZnS. At 5 kg ha⁻¹ ZnS, the maximum ZnCG (67.5 mg kg⁻¹) was achieved with 7.8 kg ha⁻¹ of ZnF. At 10 kg ha⁻¹ of ZnS, the maximum ZnCG (66.0 mg kg⁻¹) was obtained with 4.9 kg ha⁻¹ ZnF. These ZnCG values in common bean were higher than the targeted 56 mg kg⁻¹, aimed by the HarvestPlus program (White & Broadley, 2011). These results indicate that high rates of ZnF up to 10 kg ha⁻¹ ZnSO₄·7H₂O do not cause leaf scorch in common

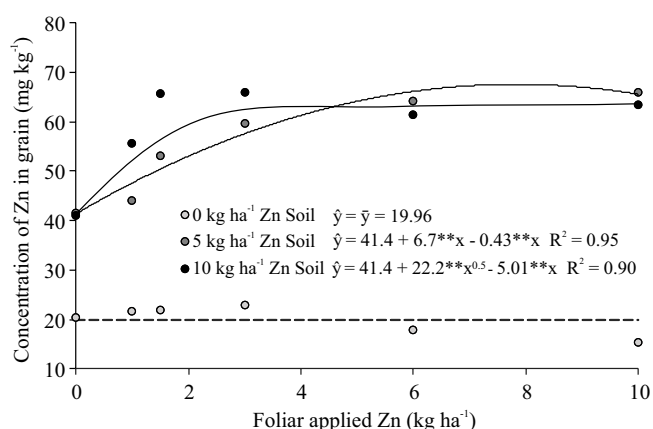


Figure 2. Interaction between rates of Zn applied to soil and rates of Zn applied to foliage of common bean (*Phaseolus vulgaris*) on Zn concentration in grain, in a greenhouse experiment.

bean, at least in the environmental conditions where the present experiment was conducted.

Studies with rice and wheat suggest that the increase in ZnCG is generally much higher with ZnF than with ZnS (Cakmak & Kutman, 2018). Our results differ from those obtained with cereals, since, in common bean, ZnS increased the ZnCG proportionally more than ZnF. However, additional studies are necessary to confirm the results of the present study with common bean plants without Zn deficiency, especially under field conditions. ZnF has a lower effective cost than ZnS, since ZnS react with soil decreasing Zn availability to plants (Joy et al., 2015). In addition, ZnF may be mixed with fungicide or insecticide (Ram et al., 2016), reducing the cost of fertilization. ZnS and ZnF combination is complementary to genetic biofortification, a long-term strategy to select genotypes with high capacity to store Zn in grains (Beebe et al., 2000).

ZnS, but not ZnF, reduced the P concentration in grains from 21.4 (zero Zn) to 16.6 (5 kg ha⁻¹ Zn) and 15.8 g kg⁻¹ P (10 kg ha⁻¹ of Zn) ($p = 0.031$). In a Zn-deficient soil, a biofortification study with wheat showed that both ZnF and ZnS reduced shoot and grain P concentration. Phytate (responsible for the storage of P in plant tissues) complexes Zn, which reduces its bioavailability. Therefore, the reduction of phytate in grains by fertilizing the plants with Zn means that Zn becomes more bioavailable in grains (Cakmak, 2008), which is desirable when the goal is to increase the bioavailability of Zn in food. In the present study, both forms of fertilization with Zn did not affect the concentration of K (mean = 7.81 g kg⁻¹, $p = 0.091$), Ca (mean = 1.93 g kg⁻¹, $p = 0.450$), Mg (mean = 1.65 g kg⁻¹, $p = 0.330$), S (mean = 5.42 g kg⁻¹, $p = 0.169$), and Fe (mean = 21.3 mg kg⁻¹, $p = 0.163$) in grains. Similar grain concentrations of Ca and Mg, found in the present study were also found by Ariza-Nieto et al. (2007) in common bean; however, grain concentrations of K and Fe, in the present study were lower than those found by these authors, especially for Fe.

Energy-dispersive X-ray spectroscopy (EDX analysis) of grain with three ZnCG indicate, by colors (Figure 3), the content of Zn in fractions of the grains: blue, low Zn; green, more Zn than the sites marked with blue; yellow, more Zn than the sites marked with green; and red, high Zn. In general, the embryo axis showed the highest Zn content, but high

concentrations of Zn were also observed in parts of the cotyledon. Concentration of Zn in the cotyledon may range between 17 (Cvitanich et al., 2011) and 41 mg kg⁻¹ (Blair et al., 2013), and Zn concentrates in the provascular region of the cotyledon (Cvitanich et al., 2011). Since, the cotyledon represents 88–91% of the bean weight (Ariza-Nieto et al., 2007), this fraction of seed contains between 83 and 94% of the total Zn in the grain (Cvitanich et al., 2011). The concentration of Zn in the embryonic axis may range from 53 to 80 mg kg⁻¹ (Cvitanich et al., 2011), but this organ represents 0.6–1.8% of the bean weight (Ariza-Nieto et al., 2007). Grain coat represents 7.6–9.7% of the bean weight (Ariza-Nieto et al., 2007), and the Zn concentration in this grain fraction may vary from 5 (Cvitanich et al., 2011) to 54.5 mg kg⁻¹ (Blair et al., 2013). In wheat, the content of Zn in the embryo and in the aleurone layer is much higher than in the endosperm (Cakmak et al., 2010; Cakmak & Kutman, 2018). Unlike rice and wheat, in which the edible part is

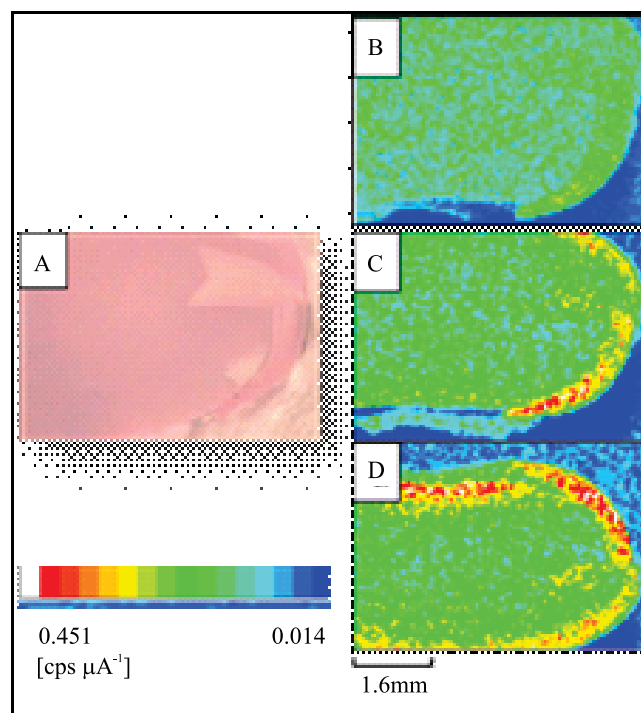


Figure 3. Grain fraction of common bean (A) used to generate energy-dispersive X-ray spectrometer images. Grain fractions were taken from samples with an average Zn concentration of 22 (B), 61 (C) and 87 mg kg⁻¹ (D); cps, count of photons per second arriving at the detector, as μA^{-1} .

usually the endosperm, common bean does not require any processing to be edible. Compared to polished rice grains, common bean have two times more Zn in grains (Pfeiffer & McClafferty, 2007). These results show the great potential of common bean to contribute to the reduction of Zn deficiency in countries where its consumption is high.

EDX analysis had already been validated for the selection of rice and millet genotypes with high Zn and Fe (Paltridge et al., 2012). Our results with common bean support this validation. In addition to be a fast and inexpensive method, EDX has these advantages: it is non-destructive; it does not use toxic chemicals; and it is performed with solid samples. EDX is a semi-quantitative method and a possible lack of sample homogeneity is the main disadvantage of this method (Paltridge et al., 2012).

Determining the appropriate fertilizer management to increase the grain-Zn concentration is an advance in the agronomic biofortification of agricultural crops. However, quantifying the bioavailability of the accumulated Zn is critical, considering that Zn in any organ of the plant is only partially available to the human organism due to antinutritional compounds such as phytates and polyphenols. Therefore, in future studies aiming at investigate Zn-enriched common bean grains, these antinutritional compounds should be quantified, and the bioavailability of Zn and Fe should be assessed.

Conclusions

1. The split application of Zn fertilizer into two or three applications on foliage, at different reproductive stages of common bean (*Phaseolus vulgaris*), to increase the Zn concentration in grains is not advantageous over a single foliar application of Zn at the pod filling stage.

2. The combined Zn application to soil and leaves increases the concentration of Zn in grains of common bean, in greenhouse.

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