

Article

Efficiency of Nanoparticle, Sulfate, and Zinc-Chelate Use on Biomass, Yield, and Nitrogen Assimilation in Green Beans

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Abstract: The introduction of nanofertilizers (Nfs) in agriculture has allowed the development of new technologies that enhance the productivity of crops. Within the most studied Nfs we find metal oxides, especially ZnO; however, the results of various experiments provide contradictory data on the growth variables. Therefore, this study intended to evaluate the efficiency associated with the use of nanoparticles, sulfates, and zinc-chelates in *Phaseolus vulgaris* L. cv. Strike grown in acid soil, as well as to evaluate its production, total biomass, and nitrogen assimilation. *Phaseolus vulgaris* L. cv. Strike plants were sprouted and grown in polyethylene bags containing 3 kg of acid soil (pH 6.8) in an experimental greenhouse and were watered with a nutritious solution. A completely randomized design including ten treatments and five repetitions was used. Treatments consisted of applying different zinc sources (sulfate, DTPA chelate, and zinc oxide nanoparticles) to four different doses (0, 25, 50, and 100 ppm of zinc). Results obtained indicated that the doses best favoring an increase in biomass, production, and nitrogen assimilation were 50 ppm of ZnSO₄, 100 ppm of DTPA-Zn, and 25 ppm of zinc oxide nanofertilizers (NfsOZn). Hence, the dose containing 25 ppm of NfsOZn was the most efficient dose, since at a lower dose it was able to equalize biomass accumulation, production, and nitrogen assimilation as compared to ZnSO₄ and DTPA-Zn sources. However, further research is required, given that high-concentration doses were toxic for beans. Finally, it is worth highlighting that zinc oxide nanoparticles have a huge potential to be used as nanofertilizers if applied in optimal concentrations.

Keywords: *Phaseolus vulgaris* L.; nanofertilizers; efficiency; fertilization; micronutrients

1. Introduction

Nanotechnology is an emerging science aimed at understanding and creating materials, devices, and systems to exploit the nanoscale properties of various materials [1]. This area of research in modern materials science is able to provide innovative and varied technological applications ranging from sophisticated medicinal techniques to food processing and agricultural production [2]. Nanomaterials currently provide physical and chemical properties that can be useful in various areas [3]. In farming, nanotechnology has developed different products to help improve soil fertilization, reduce the occurrence of diseases, and increase crop quality and production [4].

The materials with diameters that range from 1 to 100 nm and which can provide one or more nutrients to plants are known as nanofertilizers (Nfs). In comparison with conventional fertilizers, Nfs are expected to improve crop growth and productivity, as well as to reduce losses and minimize environmental impact [5].

Studies done using zinc oxide nanofertilizers (NfsOZn) have shown an increase in production, biomass, root, germination, and chlorophyll concentration [4]; however, their excessive use can produce stress in plants, affecting protein, carbohydrate, and DNA synthesis [6]. Thus, it is of paramount importance to conduct studies comparing the positive effects of Nfs vs. conventional fertilizers [5].

Zinc is considered to be one of the eight essential micronutrients for plants. It is necessary in small amounts and is crucial for appropriate development [7], since it promotes an increase in biomass and enzymatic action. Moreover, it stabilizes chlorophyll molecules and regulates gene expression [8,9].

The common bean (*Phaseolus vulgaris* L.) is one of the most important grain legumes for human consumption around the world [10]. The green bean (fresh pod bean) has a high nutritional value due to its high mineral, fiber, protein, carbohydrate, and vitamin content [11]; however, this plant is highly prone to showing zinc deficiency. The Strike green bean is a variety that in several studies has shown a lower yield and quality compared to other cultivars [11,12]; NpsOZn are an alternative to increase the quality and production of this variety.

Therefore, this study intended to evaluate the efficiency associated with the use of nanoparticles, sulfates, and zinc-chelates in *Phaseolus vulgaris* L. cv. Strike grown in acid soil, as well as to evaluate its production, total biomass, and nitrogen assimilation.

2. Materials and Methods

2.1. Crop Management

Phaseolus vulgaris L. cv. Strike bean plants were sprouted and grown in polyethylene bags containing 3 kg of soil (sandy loam, pH 6.8, electrical conductivity 0.51 dS m^{-1} , organic matter 1.5%, water saturation 48%, 0.22 ppm Zn, 0.4 ppm Cu, 5.4 ppm Fe, and 1.3 ppm Mn) in an experimental greenhouse located in Chihuahua City, Mexico, at a mean temperature of $30 \pm 5 \text{ }^\circ\text{C}$.

A nutritious solution containing 6 mM of NH_4NO_3 , 1.6 mM of K_2HPO_4 , 2.4 mM of K_2SO_4 , 4.0 mM of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.4 mM of MgSO_4 , 2 μM of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.0 μM of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 μM of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.3 μM of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, and 0.5 μM of H_3BO_3 prepared with distilled water was applied [13]. The pH of the solution ranged from 5.5 to 6.0. The solution was applied until field capacity (24 mL per 100g of soil) every third day. The treatment application began 15 days after germination and was done using 200 mL of the zinc solution for each plant (once per week).

2.2. Experimental Design and Treatments

A completely randomized design including ten treatments and five repetitions was used. In Table 1, the sources and doses applied for each treatment are shown.

Table 1. Description of treatments (dosage and form of application).

Treatments	Doses (mg kg^{-1})	Source of Zn
Control	0	
S25	25	Sulfate
S50	50	
S100	100	
Q25	25	
Q50	50	DTPA chelate
Q100	100	
N25	25	
N50	50	Oxide
N100	100	

2.3. Nanofertilizer Characterization

The material applied as Nfs was zinc oxide obtained by wet chemistry methodology in the form of wurtzite crystals with an average size of 50 nm with no contaminants, a purity level of 99.7% (Figure 1), and a density of 5.61 g cm^{-3} . Figure 2 shows the morphology of the sample by scanning and transmission electron microscopy. The material was provided by the company “Investigación y Desarrollo de Nanomateriales S.A. de C.V.”.

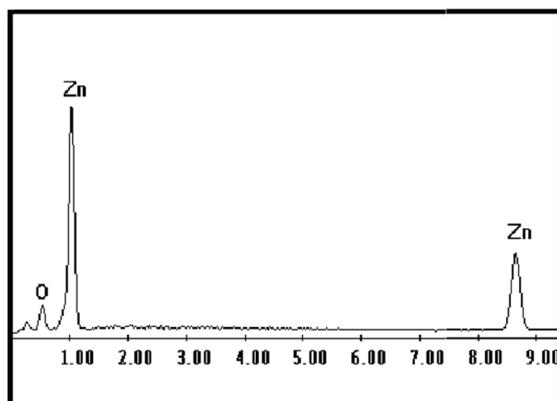


Figure 1. Elemental analysis (chemical composition) of zinc oxide nanofertilizers (NfsOZn) by energy dispersive X-ray (EDX).

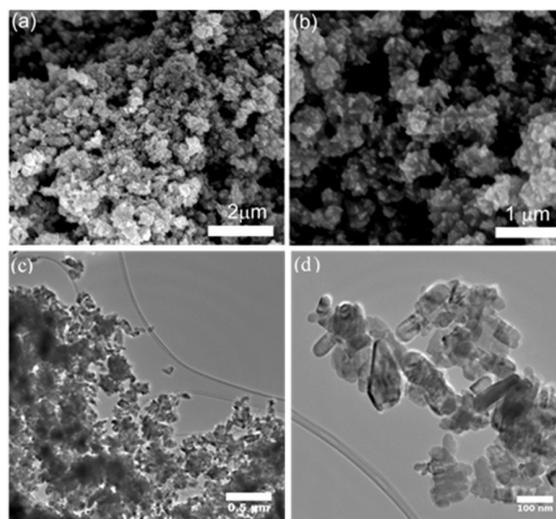


Figure 2. (a,b) NfsOZn morphology using electron microscopy scans. (c,d) NfsOZn morphology using electron microscopy transmission.

2.4. Plant Sampling

Sixty days after germination (dag), full plants were sampled. The plants were at the phenological phase of full development and maturity of the fruit. The fresh material was used to quantify biomass, yield, and photosynthetic pigments, while the dry material was used to determine zinc content. Four repetitions per treatment were used for each variable analyzed.

2.5. Plant Analysis

2.5.1. Biomass

Leaf biomass production was obtained from the average weight per plant based on dry material (g).

2.5.2. Yield

Plant yield was expressed as the mean fruit fresh weight per plant. Green beans collected from each plant were weighed at sampling. Commercial yield represents fruits of acceptable standards, while fruits lacking these qualities are represented by non-commercial or residual yield. Total yield (g per plant) is the sum of both types of yield.

2.5.3. Assay and Determination of the In Vivo Enzymatic Activity of Nitrate Reductase

The in vivo Nitrate Reductase (NR) activity (EC 1.6.6.1) was determined by the assay [14]. Leaf blades were cut into 5 mm sections (100 mg) and placed in 10 cm³ of incubation buffer (100 mM K-phosphate buffer, pH 7.5, and 1% (v/v) propanol). The samples were infiltrated and the intracellular spaces of the tissues were flushed with buffer using a vacuum (0.08 MPa). After 5 min, the vacuum was released and the samples were re-evacuated, incubated at 30 °C in darkness for 1 h, and finally placed in a boiling water bath to stop the NR activity. The resulting nitrite concentration was determined by spectrophotometry at 540 nm in a reaction mixture containing 2 cm³ of extract, 2 cm³ of 1% (m/v) sulfanilamide in 1.5 M HCl, and 2 cm³ 0.02% (m/v) *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 0.2 M HCl (NR+NO₃⁻), following the same method but using a modified incubation buffer containing 50 mM KNO₃. The NR induced by NO₃⁻ and Mo (NR+NO₃⁻ + MO), and the NR induced by NO₃⁻ and Mo (NR+NO₃⁻ + Mo), were also determined using a modification of the incubation buffer containing 20 mM NaMoO₄ and 50 mM KNO₃ plus 20 mM NaMoO₄, respectively. The resulting nitrate concentration was also determined by spectrophotometry.

2.5.4. Photosynthetic Pigments

The method used for leaf pigment extraction and quantification was the method described by Wellburn, 1994 [15], which required weighing 0.2–0.3 g of fresh photosynthetic plant material (leaves) in 7 mm diameter foliar disks. Ten milliliters of pure methanol (CH₃OH) was added. Samples were then incubated at room temperature in darkness for 24 h. After this period elapsed, absorbance was measured at 470 nm (carotenoids), 653 nm (chlorophyll b, chl b), and 666 nm (chlorophyll a, chl a). The pigment concentration calculation was carried out according to the formulas below [15].

$$\text{Chl a} : [15.65 (A_{666}) - (7.34 (A_{653}))] \frac{(\text{Chl a})(V_1)(p_1)}{(p_2)(2\pi r^2)(n)} \quad (1)$$

$$\text{Chl b} : [27.05 (A_{653}) - (11.21 (A_{666}))] \frac{(\text{Chl b})(V_1)(p_1)}{(p_2)(2\pi r^2)(n)} \quad (2)$$

where V_1 is the extraction volume, p_1 is the weight in g per foliar disk (7 mm in diameter), p_2 is the total weight in g, n is the number of foliar disks (7 mm in diameter), and r^2 is the radius of the foliar disks. The sum of chlorophyll a and chlorophyll b concentrations resulted in total chlorophyll, which was expressed in $\mu\text{g cm}^{-2}$.

2.5.5. Nitrogen Determination

A subsample of 0.1 g dry weight was digested with sulphuric acid and H₂O₂, according to Wolf [16]. After dilution with deionized water, a 1 mL aliquot of the digest was added to the reaction medium (5% potassium sodium tartrate, 100 μM sodium phosphate, and 5.4% (w/v) sodium hydroxide) with 15/0.03% (w/v) sodium salicylate/sodium nitroprusside and 5.35% (v/v) sodium hypochlorite. The samples were incubated at 37 °C for 45 min and organic N was measured by spectrophotometry at A 630 as performed by Baethgen and Alley [17]. The results were expressed as a percentage.

2.5.6. Zinc Content Determination

The Zn concentration was determined by an Inductive Coupled Plasma Optical Emission Spectrometer (Agilent Technologies 700 Series ICP-OES, CA, USA), according to the method described by Karacan and Aslantas [18]. The Zn concentration was expressed in mg kg^{-1} of dry weight.

2.6. Statistical Analysis

All data were subject to analysis. The LSD test (95%) was used to determine the difference between the means of treatment [19]. The significance levels of both tests are expressed as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and NS (not significant).

3. Results and Discussion

3.1. Biomass and Production

The studies done using zinc application showed an increase in the productivity of various crops [20]. This experiment found that the best doses for each fertilizer applied were as follows: 50 mg kg^{-1} for ZnSO_4 , 100 mg kg^{-1} for DTPA-Zn, and 25 mg kg^{-1} for NfsOZn, which increased production by 13.7, 20.4, and 15.5 g per plant, respectively, compared to the control reference, to which no fertilizer was applied (Figure 3). These data concur with those reported by Pérez-Álvarez et al. [21] and Weldua et al. [22], who identified an increase in production after applying zinc. In NfsOZn, low doses were observed to work better than high doses, which may be due to the fact that Nfs are smaller than 100 nm, which facilitates penetration in plants [23]. However, it is worth noting that high NfsOZn doses (400 mg kg^{-1}) may affect root development in plants [24].

Zinc deficiency in plants affects photosynthesis due to altered chloroplast pigments [25]. The most visible zinc deficiency symptoms are short internodes, a decrease in leaf size, and delayed maturity [26].

Mankad et al. [27] mentioned that growth enhancement by ZnO NPs may be attributed to the release of Zn^{2+} ions for greater plant growth. The same authors concluded that nanoparticles, if applied at optimum concentration, can enhance the growth of plants both morphophysiologically as well as biochemically.

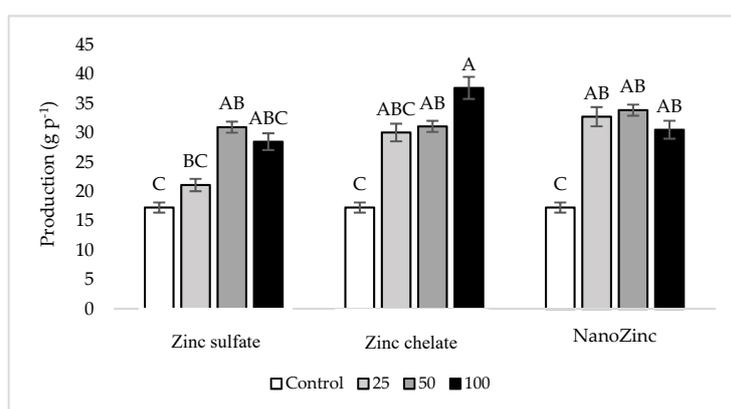


Figure 3. Effect of applying nanofertilizers, chelates, and zinc sulfates in green bean production per plant. Letters show significant differences.

A number of studies have shown that biomass accumulation is a basic parameter by which fertilization efficiency in crops can be evaluated [28]. This study found significant differences in biomass accumulation due to the effects caused by applying different sources of zinc. The doses that showed greater biomass accumulation were 25 ppm of NPsOZn, 50 mg kg^{-1} of ZnSO_4 , and 100 ppm of Zn-DTPA at 42%, 35%, and 45% increments, respectively, compared to the control with no fertilizer application (Figure 4). These data align with those reported by Sida-Arreola et al. [29]

and Pérez-Álvarez et al. [21], who identified that zinc application in the form of chelates and sulfates increased the biomass of common beans. A number of studies done with various crops showed that NfsOZn application increases plant growth and development [30].

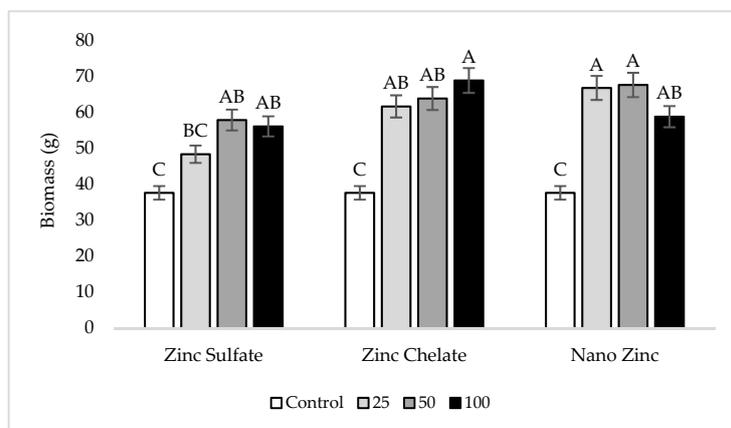


Figure 4. Effect of applying nanofertilizers, chelates, and zinc sulfates to the total biomass of green beans cv. Strike. Letters show significant differences.

3.2. *In Vivo* Nitrate Reductase Activity

NR is the first enzyme participating in nitrogen assimilation and its activity is regulated by different environmental stimuli, such as nitrate and molybdenum content and the ratio between sugar and amino acid content [31,32].

Treatments with zinc application were observed to induce increased NR enzyme activity as compared to the control treatment (Figure 5). The best doses were 25 mg kg⁻¹ of NfsOZn, 50 mg kg⁻¹ of ZnSO₄, and 100 mg kg⁻¹ of Zn-DTPA. These results are consistent with the data obtained for the biomass and production variables.

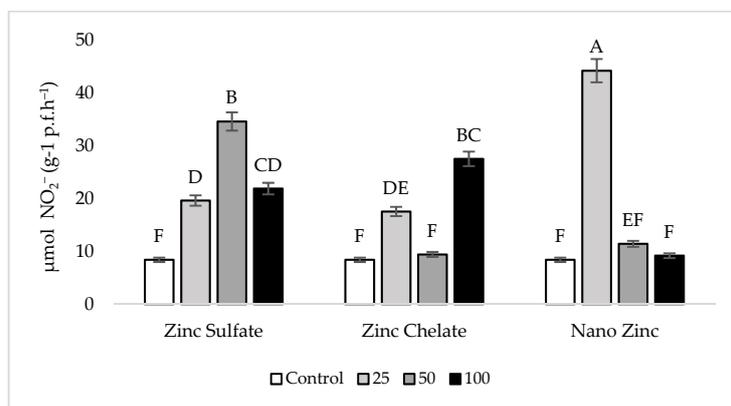


Figure 5. Endogenous nitrate reductase activity induced by nitrates due to the effect of applying nanofertilizers, chelates, and zinc sulfates in green beans cv. Strike. Letters show significant differences.

Zinc is considered to be one of the eight essential micronutrients for plants. It is necessary in small amounts and crucial for appropriate development [7], since it promotes an increase in biomass and enzymatic action. Moreover, it stabilizes chlorophyll molecules and regulates gene expression [8,9]. Additionally, it has been observed that zinc is required for various types of enzyme activity, carbohydrate metabolism, and protein synthesis [33]. Zinc is an essential micronutrient for biological systems and plays a crucial physiological role in protein synthesis and metabolism. It is also a structural constituent and regulatory cofactor in enzymes and proteins involved in many biochemical pathways. Almost 40% of the Zn-binding proteins are transcription factors needed for

gene regulation, and 60% are enzymes and proteins involved in ion transport. Zinc plays an important role in increasing leaf iron concentration and also is an integral part of many enzymes and proteins [34]. Regarding the latter, an important relationship was found between the nutritional status of zinc and N assimilation, showing an important concentration of Zn ions, which favors the *in vivo* enzymatic activity of nitrate reductase. However, as occurred with regard to the effect obtained in biomass accumulation and production, the efficiency of the zinc application will depend on the source and doses of the application.

3.3. Content of Photosynthetic Pigments

Chlorophyll a, is the photosynthetic pigment of greater amount found in plants. It is more sensitive to degradation and is a good indicator that can be used to detect stress conditions in plants, such as those produced by applying excess metal nanoparticles [35,36]. This study found that chlorophyll content in bean leaves was similar for the treatments applying 25 ppm NfsOZn, and 25, 50, and 100 ppm DTPA-Zn, showing an increase of 83.14, 80.28, 80.5, and 77.04%, respectively, with respect to the control (Figure 6). It was observed that as the dose of NfsOZn increased, the pigment concentration was lower. This result concurs with that reported by Du et al. [35], who mentioned that the application of high doses to the soil may cause toxicity.

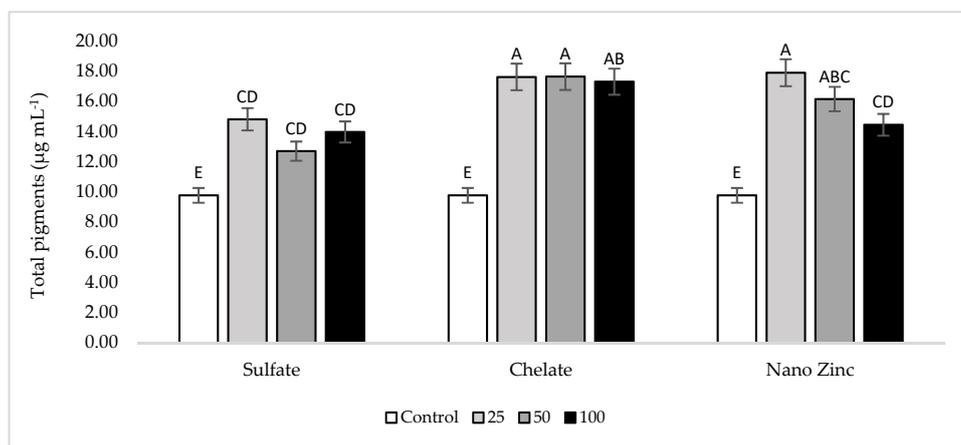


Figure 6. Total pigment concentration in leaf of bean cv. Strike as a result of applying nanofertilizers, chelates, and zinc sulfates. Letters show significant differences.

Samreen et al. [36], while evaluating the effect of zinc application on rate of growth as well as on chlorophyll and protein, concluded that applying 2 µM to the nutritious solution improved the plant growth of *Vigna radiate*, chlorophyll content, raw protein, and zinc content.

3.4. Zinc Content

Zinc is absorbed in very small amounts by plants in the form of a divalent cation (Zn^{+2}), and zinc availability is greater when the pH is lower. Zinc concentrations lower than 20 mg kg⁻¹ in dry matter can cause severe deficiencies in crops [37]; however, crops can have different tolerance ranges. Certain species like soybean, rice, and beans are most sensitive to zinc deficiency [38].

In this experiment, it was observed that ZnSO₄ application increased zinc concentration in green beans. The 100 mg kg⁻¹ application yielded better results in the root, leaf, and fruit, having shown an increase of 31, 44.5, and 5 mg kg⁻¹, respectively (Figure 7).

When applying DTPA-Zn, the 25 mg kg⁻¹ dose was observed to accumulate a greater concentration in the leaf and root, while the 100 mg kg⁻¹ dose did so in the fruit and stalk (Figure 7). Concentrations in tissues were found in the following order: stalk, root, leaf, and fruit.

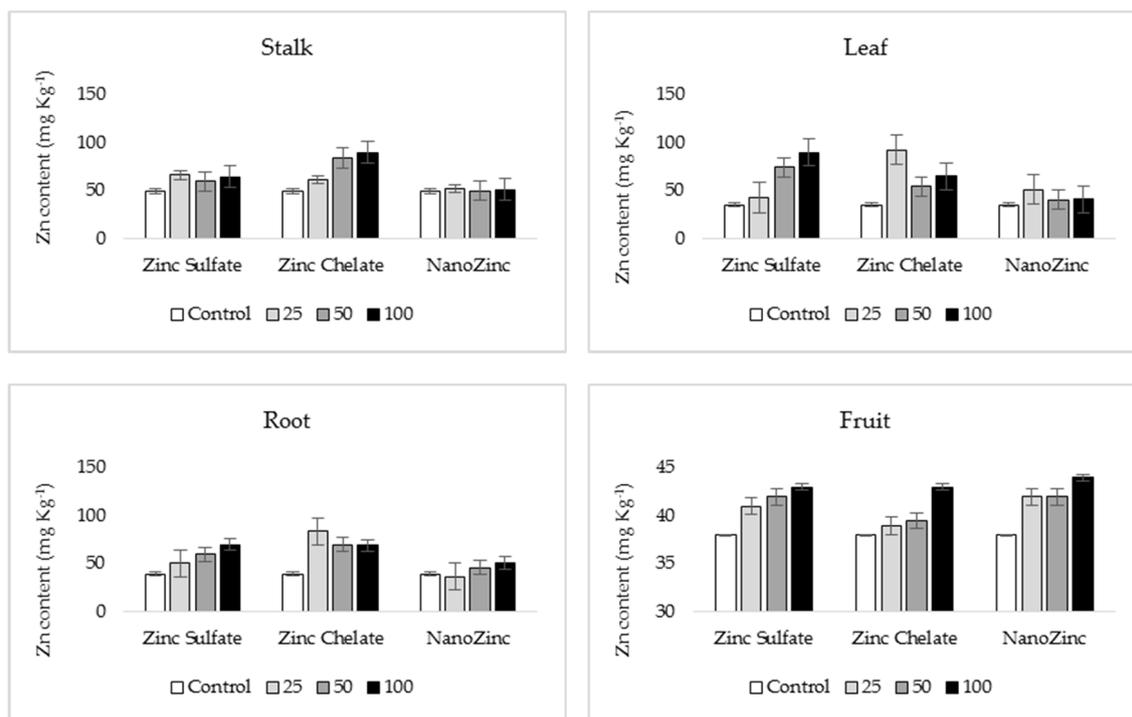


Figure 7. Zinc concentration in the root, stalk, leaf, and fruit of green beans cv. Strike in response to the application of nanofertilizers, chelates, and zinc sulfates.

NfsOZn showed an increase in zinc concentration compared to the control treatment applied to the root, leaf, and fruit; however, the results seen in the stalk were similar to those shown by the control treatment (Figure 7). The NfsOZn treatment was observed to show the highest zinc concentration in the fruit, which suggests that NfsOZn can help in the biofortification of cv. Strike green beans.

In general, there is limited information on the effect that NfsOZn have on the physiological and biochemical parameters of beans. Therefore, it is necessary to conduct a more in-depth evaluation of the effect caused by nanoparticles (nanofertilizers) on the physiology and biochemistry of various farm crops, as well as the impact thereof on the environment. Moreover, a negative effect has been observed for high zinc doses over the parameters studied, such as biomass accumulation, yield, photosynthetic pigment content, and the *in vivo* activity of nitrate reductase. Hence, it is recommendable to study this. In this regard, Wang et al. [39] mentioned that zinc is a necessary element for plants, but excess Zn can be detrimental. The same authors stated that excess Zn exerts its toxicity partially through disturbing the nutrient balance and inducing oxidative stress in plants. These data will be helpful for better understanding of toxicity of Zn and the adaptive mechanism in Zn non-hyperaccumulator plants.

4. Conclusions

The best doses favoring an increase in biomass, production, and nitrogen assimilation were 50 ppm of ZnSO₄, 100 ppm of DTPA-Zn, and 25 ppm of NfsOZn. Hence, the dose containing 25 ppm of NfsOZn was the most efficient dose, since at a lower dose it was better able to equalize biomass accumulation, production, and nitrogen assimilation as compared to ZnSO₄ and DTPA-Zn sources. The efficiency of the zinc application depended on the source and doses of the application. Further research is required, given that high NfsOZn doses were toxic for beans. Finally, it is worth highlighting that zinc oxide nanoparticles have a huge potential to be used as nanofertilizers if applied in optimal concentrations.

Author Contributions: E.S. and C.O.P.-G. designed the study. J.M.S.-P. and R.P.-L. analyzed the data. E.S. and C.O.P.-G. prepared the manuscript, while E.M.-M., F.J.P.-R., R.P.-L. and R.M.Y.M. conducted the experiments. C.O.P.-G., J.M.S.-P. and E.S. organized the data and performed the statistical analysis. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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