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# Initial Development of Corn Seedlings after Seed Priming with Nanoscale Synthetic Zinc Oxide

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**Abstract:** Nanofertilizers are increasingly explored for sustainable micronutrient delivery in agriculture. Pre-treating seeds with nanofertilizers prior to planting (i.e., seed priming) reduces concerns about nanoparticle (NP) fertilizer non-target dispersion; however, priming formulations and concentrations must be carefully selected to avoid germination inhibition and toxicity. Here we investigate changes in corn seed germination and seedling development after seed priming with ZnO NPs, ZnO bulk and ZnCl<sub>2</sub>. To evaluate the effects sterile seeds were immersed in priming solutions of 0, 20, 40, 80, 160 mg L<sup>-1</sup> Zn for the three Zn sources. Following an 8 h priming the seeds were evaluated for germination and vigor for 5 days on germination paper. Root and shoot lengths were measured as well as fresh and dry biomass. Compared to the control, the ZnO NP and ZnCl<sub>2</sub> seed priming promoted beneficial effects. ZnO NP seed-priming exhibited a concentration dependent profile in improving seedling growth, with greatest benefit around 80 mg L<sup>-1</sup>, providing 17%, 25% and 12% higher values than control for germination, root length, and dry biomass production, respectively. In contrast, seeds primed with bulk ZnO did not differ from the control. These findings support NP-seed priming as an alternative to delivery of essential micronutrients, such as zinc, to corn seedlings.

**Keywords:** nano-fertilizer; seed-priming; phytotoxicity; characterization; XRD; TEM

## 1. Introduction

Nanoparticle (NP) engineering is a promising a tool for several areas, including food science and agriculture. Applications in these areas have been gaining prominence, mainly regarding food biofortification to supply or alleviate human nutritional deficiency [1]. Nanotechnology shows positive prospects for sustainable agricultural practices and is expected to be a nano-tool for increasing yields minimizing non-target agrochemical distribution and reducing waste [2,3].

While nanotechnology applications are now commonplace in many industries, the application and effects of NPs in agriculture is still very incipient [1]. A NP is any material having at least a single dimension between 1 and 100 nm [4]. NPs are often extremely reactive due to of their high specific surface area (30–50 m<sup>2</sup> g<sup>-1</sup>), promoting catalytic activity and rapid chemical reactions, which can lead to positive and negative impacts when applied as nanofertilizers in complex biological systems [5].

Among NP fertilizers, the micronutrient Zn, delivered using nano-scale ZnO has been widely evaluated. However, often, the results on the beneficial and detrimental effects of these applications

can be quite contrasting [6]. For example, 400 or 800 mg kg<sup>-1</sup> ZnO NPs reduced dry biomass and corn yield [7], although the application of 500 mg kg<sup>-1</sup> of ZnO NP stimulated the development of soybean [8]. Contrasting results are often due to differing experimental conditions (hydroponics, sand, soil, soil type, application method, crop, time etc.). Thus, the outcomes from NP-plant are often highly specific to experimental conditions, making it challenging to extrapolate findings to field applications with crops of economic interest.

Zn is one of the microelements essential to the plant life cycle, being found in all enzymes related to the metabolism of plant oxyreductase, lyases, isomerases, transferases, hydrolases and ligases [9]. Zn also increases the biosynthesis of chlorophylls and carotenoids, consequently, stimulating the entire photosynthetic system and the incorporation of carbon by plants. In addition, this element is precursor of the important tryptophan amino acid in the synthesis of the hormone auxin responsible for plant growth and development. Zn also exhibits antimicrobial properties that may be also exploited to increase crop yield under biotic stress and can induce the synthesis of antioxidant enzymes in plants including catalase and peroxidase [10]. However, soil Zn deficiency has been reported as the most widespread deficiency among the micronutrients [11].

ZnO NP application can be performed via soil [6], foliar [12] and seed priming [13] and positive effects have been reported since the seed germination until harvest in low concentration [14]. When ZnO NP application is carried out via seeds, the particles can penetrate through the seed coat and move through the conductive tissues, being absorbed, translocated and accumulated in tissues during plant development. NPs also may enter via stomata in leaves, although much of the application is to soil delivery [15]. NP mobility, modification and bioavailable in soil is highly dependent on soil properties such as pH, being higher dissolution in lower pH [16]. Therefore, seed priming is a target delivery approach to increase the availability of micronutrient during early growth. The direct delivery of the NP to the seed prior to planting also removes much of the experimental variability.

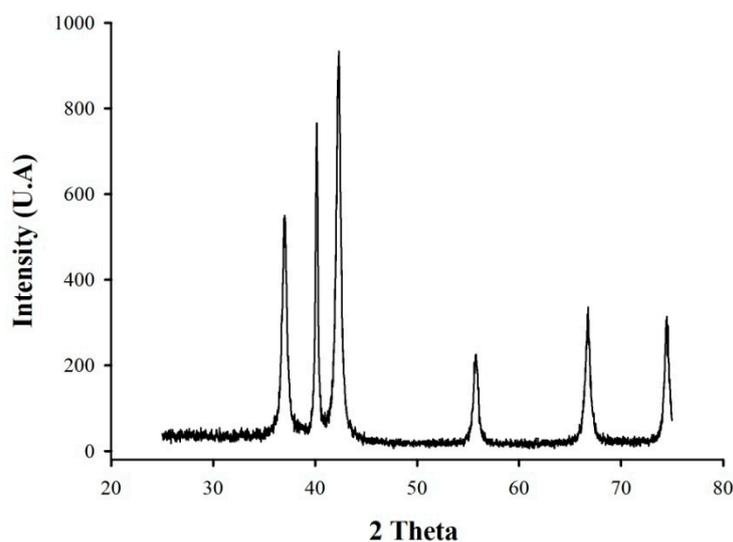
Among the crops of greatest world interest, corn is a demanding crop in Zn, and this element in turn is the micronutrient with the greatest possibility of becoming limiting to the growth and development of corn. However, the amount of zinc required by the corn crop is relatively low, making it difficult to apply uniformly at the target concentration, so the application of Zn via seed treatment is an interesting management alternative [17].

Therefore, it was hypothesized that Zn sources, differing in solubility and size, present specific responses in corn seedlings development, when applied through seed priming, since in corn, zinc deficiency symptoms are usually seen in the first days of the plant's life. In this context, the specific aim was to investigate changes in the growth and development of corn seedlings after application of increasing concentrations of Zn as ZnO NP, ZnO bulk and soluble source zinc chloride (ZnCl<sub>2</sub>). In addition, if there is a possibility of reducing Zn concentrations applied to avoid unnecessary losses of the available sources when using ZnO NP possible toxic effects of ZnO NPs would also be greatly reduced.

## 2. Materials and Methods

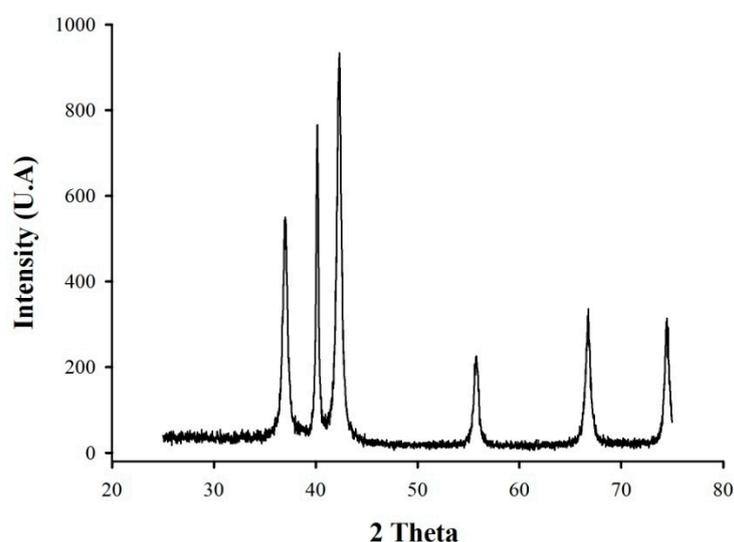
The research was carried out in the fertilizer laboratory of the Soil Study Group (GESSO-UEM). ZnO NP were prepared using as reaction precursors the solutions of 100 mL of ZnSO<sub>4</sub> · 7H<sub>2</sub>O (purchased from Sigma Aldrich Chemical Co<sup>®</sup>, San Luis, MO, USA) 0.1 mol L<sup>-1</sup> and 100 mL of 0.4 mol L<sup>-1</sup> NaOH (Sigma Aldrich Chemical Co). The solutions were prepared, and then slowly mixed in a 500 mL Erlenmeyer flask. After that, the solution was shaken vigorously on a magnetic stirrer at 1500 rpm at room temperature (22 °C ± 2) for 15 min uninterrupted. The reaction flask was placed in a microwave oven for 2 minutes at a power of 700 W. The reaction flask was cooled at room temperature (22 °C ± 2). After formation of the white precipitate, the flask was centrifuged and washed several times with ultrapure water to remove excess SO<sub>4</sub><sup>2-</sup> and Na<sup>+</sup> residuals from the reaction. The solution was frozen instantly at -70 °C using liquid nitrogen and dried by sublimation in a LS 3000 lyophilizer (Terroni<sup>®</sup>, São Carlos, São Paulo, Brazil) and packed in a vacuum glass desiccator, protected from light.

The ZnO powder was analyzed by X-ray diffraction in an XRD 6000 system (Shimadzu<sup>®</sup>, Kyoto, Japan). Diffractograms were obtained between 20 to 75° 2 $\theta$ , in intervals of 0.02° 2 $\theta$  for 0.6 s, step-mode, using CoK $\alpha$  radiation and nickel filter. The diffractogram peaks correspond to zincite (ZnO) (Figure 1).



**Figure 1.** X-ray diffraction of the synthetic zinc oxide nanoparticle (Zincite-ZnO NP).

The size and shape of the particles were determined by transmission electron microscopy (TEM, CM200, Philips<sup>®</sup>, Amsterdam, Netherlands). TEM samples were prepared by depositing a small volume of a ZnO in water suspension onto carbon grids coated with copper grids and allowed to dry overnight. Synthetic ZnO NP used in this assay had a diameter of approximately 20 nm and rounded shape, as can be observed at Figure 2.



**Figure 2.** TEM image of the synthetic zinc oxide nanoparticle (Zincite-ZnO NP). Scale bar is 20 nm.

The seeds of corn hybrid IAC 8046 were assigned by a company authorized to commercialize seeds (Sella Sementes<sup>®</sup>, Astorga, Paraná, Brazil). Hybrid corn seed were chosen because is worldwide cultivated and responsive to inputs added. The seeds selected were the same size in order to minimize the errors of vigor and germination. The seeds were disinfected with sodium hypochlorite solution (1% v/v) for 2 min and then washed several times with ultrapure water. Before the experiment installation, seeds were kept in a dry and dark place at a temperature of 16 °C.

To evaluate the effects of Zn sources application in corn, a cross-factor experiment ( $5 \times 3$ ) was carried out in a completely randomized design with 4 replicates. The five-level factor was concentrations (0, 20, 40, 80 and  $160 \text{ mg L}^{-1}$ ) concentrations based on Zn content and the factor with three levels were the different sources (ZnO NP,  $\text{ZnCl}_2$ , and ZnO bulk). Each plot was composed of 50 seeds that were immersed in the different solutions/suspensions containing a total volume of 200 mL with different Zn concentrations for 8 h, then dried at room temperature for one hour the pH of each solution at the start and end of the 8 h is shown in Table 1.

**Table 1.** pH values measured different solutions, before and after corn seed priming.

Concentration ( $\text{mg L}^{-1}$ )	0	20	40	80	160	0	20	40	80	160
	Initial pH					pH After 8 h				
ZnO bulk	6.50	7.45	7.53	7.56	7.60	6.23	7.38	7.42	7.45	7.56
ZnO NPs	6.50	7.67	7.70	7.76	7.81	6.23	7.64	7.68	7.69	7.73
$\text{ZnCl}_2$	6.50	6.20	6.37	6.50	6.60	6.23	6.64	6.68	6.69	6.81

The Zn NP were compared with two other Zn sources. The first was a Zn ionic solution, which was prepared by dissolving  $\text{ZnCl}_2$  purchased from Sigma Aldrich Chemical Co in ultrapure water, the second one was ZnO bulk with non-nanometric ( $>300 \text{ nm}$ ) size which was purchased from Sigma Aldrich Chemical Co. The insoluble sources (Synthetic ZnO NP and ZnO bulk) were suspended directly in ultrapure water and dispersed using an ultrasonic vibration shaker (100 kHz for 5 min).

Seed germination was assessed using Gemitest paper (Germipel<sup>®</sup>, Ribeirão Preto, São Paulo, Brazil). The seeds were placed in three sheets moistened with ultrapure water equivalent to 2.5 times the dry biomass of the paper and each seed was one cm or more away from another seed. The rolls were prepared and taken to the Mangelsdorf germination chamber set at a constant temperature of  $25 \text{ }^\circ\text{C}$  for a period of 8 days. The results were expressed as percentage of normal (perfect seeds with root and shoot well defined), abnormal (without shoot or root) and non-viable (non-germinated) seedlings. The vigor test was performed on the fifth day, counting the number of vigorous seedlings [18].

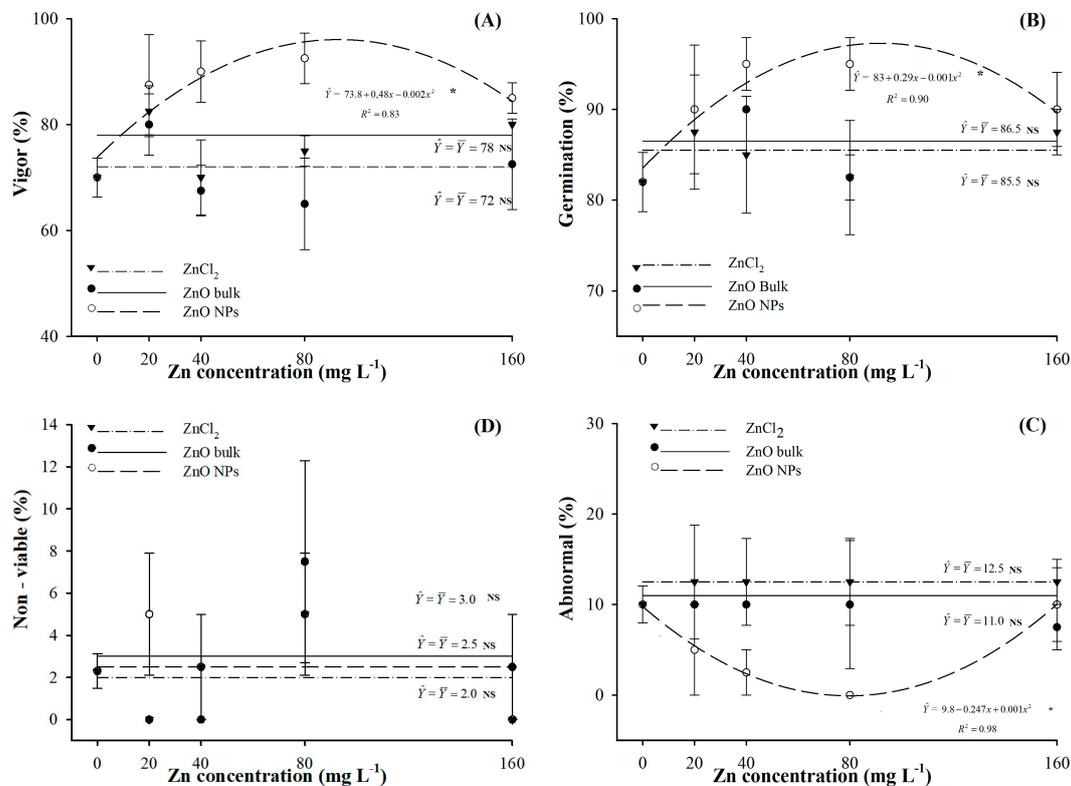
After the germination test, the root length (distance in cm from the stem base to the root tip) and shoot length (distance in cm from the base of the leaf to the tip of the leaf) were measured in 15 seedlings randomly selected with the aid of a millimeter ruler. Then, the seedlings were transferred to Kraft paper bags and weighed to obtain the fresh biomass production, later these same seedlings were taken to a drying oven at a constant temperature of  $60 \text{ }^\circ\text{C}$  for 72 h to measure the dry biomass production.

The data of all the variables were submitted to the basic statistical assumptions using the Shapiro-Wilk (normality of errors) and Bartlett (homoscedasticity of variances) tests ( $p > 0.01$ ). Subsequently, the different concentrations and sources and possible interactions were tested by means of the F test in the analysis of variance (ANOVA), when the interaction was deployed only for concentrations within each source. Quantitative data “concentrations” were analyzed by means of regression and their coefficients submitted to the t test. On the other hand, the qualitative data “sources” were analyzed by means of the Tukey average test. For all statistical interpretations, 5% probability was used ( $p < 0.05$ ) [19].

### 3. Results

The influence of nano, bulk, and ionic Zn priming sources on corn seed and seedling health vs. priming solution Zn concentrations are presented in Figure 3. Priming effects were evaluated as seedling vigor, germination percent, non-viable and abnormal seed percentages. The vigor (Figure 3A) and germination (Figure 3B) of the seeds exhibited a similar concentration trend for the ZnO NP source. For both variables, the ZnO NP application promotes an increase in germination and vigor up to a threshold concentration, after which, increasing concentrations provide diminishing benefit. It should be noted that all of the concentrations of ZnO NPs were better or equal to germination and vigor in the controls (8 h seed priming in pure water). The germination and vigor could be fit to a simple

quadratic model ( $p < 0.05$ ) predicting a maximum benefit near 120 and 145 mg L<sup>-1</sup> of Zn for vigor and germination, respectively.



**Figure 3.** Seed vigor (A), seed germination (B), non-viable seed (C), and abnormal seed (D) of corn after application of different concentrations and sources of Zn. \* = significant at 5% of probability by regression test. NS = non-significant at 5% of probability by regression test.

These maximum concentrations correspond to 95.0% for vigor and 97.0% for germination of corn seeds. However, above these concentrations, the germination and vigor of the corn seeds decreases. At the highest tested concentration of 160 mg L<sup>-1</sup>, the vigor was approximately 86% and germination was near 89%. Both of these values are still above those observed for the control corn seeds primed in a Zn-free control, which yielded 70% for vigor and 83% for germination, according to regression test ( $p < 0.05$ ).

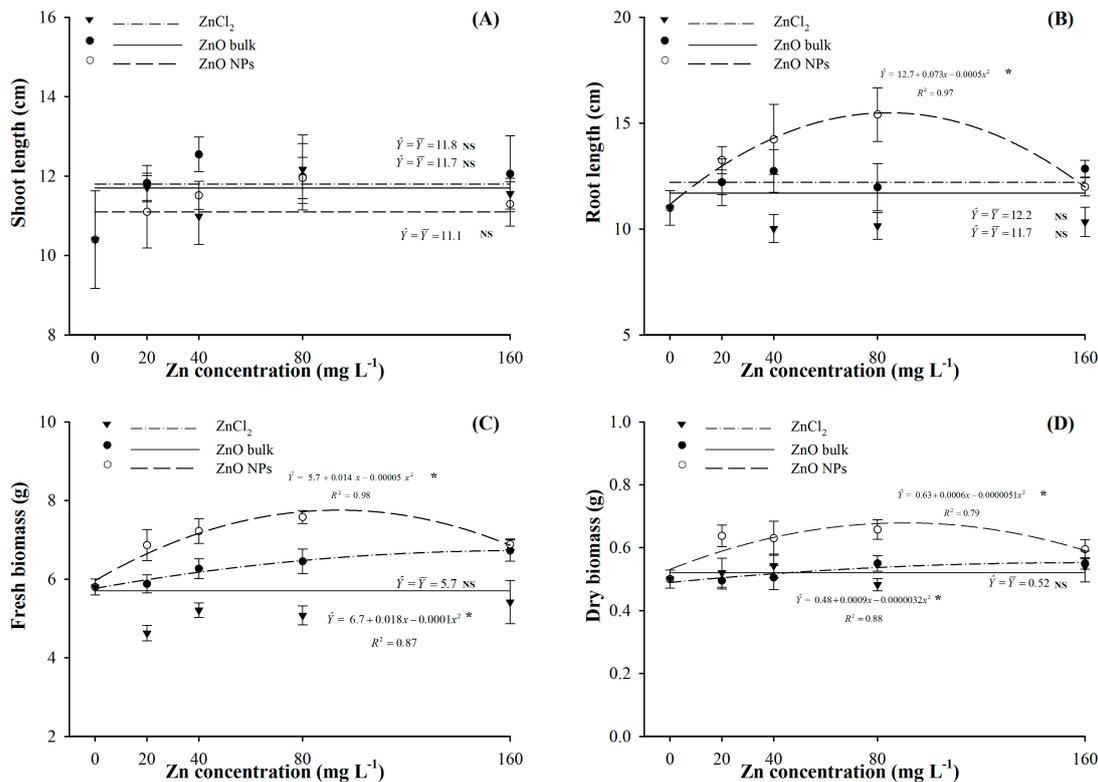
In contrast to the ZnO NPs, seeds primed with bulk ZnO or ZnCl<sub>2</sub> did not yield statistically significant differences for the germination and vigor for the Zn concentration range 0–160 mg L<sup>-1</sup> Zn. The averages of 72.0% and 78.0% of vigor, without statistical differences ( $p < 0.05$ ), and 86.5 and 85.5% of germination, without statistical differences ( $p < 0.05$ ), for ZnO bulk and ZnCl<sub>2</sub>, respectively.

Figure 3C illustrates that the ZnO NP-primed seeds showed a statistically ( $p < 0.05$ ) significant decrease in abnormal seedlings up to a concentration of 80 mg L<sup>-1</sup>, which yielded no abnormal seedlings. On the other hand, seeds primed with bulk ZnO or ZnCl<sub>2</sub> did not yield statistically significant differences in abnormal seedlings across Zn concentration range 0–160 mg L<sup>-1</sup> Zn. The averages across all concentrations of were 12.5% and 11.0% of vigor, without statistical differences ( $p < 0.05$ ) for ZnO bulk and ZnCl<sub>2</sub>, respectively.

The non-viable seeds were not significantly influenced ( $p < 0.05$ ) by Zn concentration in any of the sources tested (Figure 3D). The averages across all concentrations for non-viable seeds were 3.0%, 2.5%, and 2.0% for ZnO NP, ZnO bulk and ZnCl<sub>2</sub>, respectively.

No significant statistical differences by regression test ( $p < 0.05$ ) were observed for shoot length at the concentrations tested in any sources (Figure 4A). The averaged values calculated across all concentrations of Zn for each priming source were 11.7 cm, 11.8 cm, and 11.1 cm for ZnCl<sub>2</sub>, ZnO

bulk and ZnO NP, respectively. When evaluating for a concentration dependant trend, a significant effect was observed only for the ZnO NP-primed seed root lengths. Through a regression model ( $p < 0.05$ ) it is observed that a  $72.6 \text{ mg L}^{-1}$  Zn NP-priming concentration promote the highest root length (15.36 cm). The root length decreases for higher NP priming concentrations, however even at the highest concentration tested of  $160 \text{ mg L}^{-1}$  Zn the length was still equivalent to the control without any priming.



**Figure 4.** Seedling shoot length (A), root length (B), fresh biomass production (C), and dry biomass production (D) of corn after different concentrations and sources of Zn. \* = significant at 5% of probability by regression test. NS -non-significant at 5% of probability by regression test.

Fresh biomass and dry biomass production exhibited similar trends for the ZnO NP-primed seeds, exhibiting a maximum beneficial concentration followed by a decrease. (Figure 4C and D). For ZnO NP, the highest fresh biomass production obtained (7.51 g) was at  $90 \text{ mg L}^{-1}$ . The seeds primed with the zinc salt also showed a trend of increasing biomass with increasing concentration for the ZnCl<sub>2</sub>-primed seed, exhibited a maximum fresh biomass production of 6.72 g for  $140 \text{ mg L}^{-1}$  Zn. This trend indicates that the ZnO NP source was more efficient on a per mass basis than zinc salt.

The maximum fresh biomass production obtained (6.72 g) was at  $140 \text{ mg L}^{-1}$ , even at lower. For the ZnO NP source, the highest dry biomass production obtained (0.64 g) was predicted at  $58.8 \text{ mg L}^{-1}$ , and for the ZnCl<sub>2</sub> source the maximum dry biomass production obtained (0.54 g) was predicted at  $140.6 \text{ mg L}^{-1}$ . Even at lower concentrations, the ZnO NP source was more efficient in providing dry biomass production gains of the corn seedlings.

Table 2 shows the results referring only to the zinc sources, independent of concentrations, for all the variables evaluated in the present study. It was observed that the ZnO NP priming yielded better results for the variables vigor, germination, root length, total fresh and dry biomass production of the seedlings.

**Table 2.** Zinc sources effects, independently of concentrations on vigor, germination, non-viable and abnormal seeds, root and shoot length and total fresh and dry biomass production of corn.

Source	Vigor	Germination	Non-Viable	Abnormal	Root	Shoot	TFMP <sup>1</sup>	TDMP <sup>2</sup>
	%			cm			g	
ZnO bulk	77.5 ab	85.5 b	2.0 a	12.5 a	10.6 b	11.7 a	5.1 a	0.53 b
ZnO NPs	83.0 a	90.5 a	3.0 a	6.5 a	13.5 a	11.1 a	7.0 a	0.63 a
ZnCl <sub>2</sub>	71.0 b	86.5 ab	2.5 a	11 a	12.1 a	11.8 a	6.2 b	0.51 b
Average	77.2	87.5	2.5	10.0	12.1	11.5	6.1	0.56
CV <sup>3</sup> (%)	7.8	3.0	20.0	31.2	12.0	3.3	15.6	11.5

<sup>1</sup> = Total fresh biomass production; <sup>2</sup> = Total dry biomass production. <sup>3</sup> = coefficient of variation. Values followed by the same letter in a column do not differ from each other at 5% probability by the Tukey test (a, ab, b).

#### 4. Discussion

In the present study it was demonstrated that corn seed priming with an aqueous suspension of ZnO NP for concentrations around 70 and 100 mg L<sup>-1</sup>, promoted significant gains in germination, vigor, root length and total fresh and dry biomass production, as well as decrease in abnormal seedlings when compared to the same concentration of soluble and bulk source tested, and also to the control primed in pure water. The current results clearly indicate that ZnO NPs are effective in enhancing seedling growth and development. The higher plant growth with NPs might be due to the mobilization of nutrients as well as increase in microbial activity in the rhizosphere [20].

In relatively low concentrations, ZnO NP serves as a source of fertilizer for corn growth and development and its amount required is lower than the soluble sources such as ZnCl<sub>2</sub>. Therefore, ZnO NPs can be used when considering the same amount of Zn in the sources, since the efficiency of use by plants may be higher due to the facilitated entry of the NPs due to their size and reactivity which improve chances to cross cell walls and transport across the plasma membranes. A gradual decrease in efficiency is observed with increasing concentrations, with the highest ZnO priming solution of 160 mg Zn L<sup>-1</sup> showing no benefits compared to the non-primed seeds or seeds primed with ZnCl<sub>2</sub> or bulk ZnO. Establishing a therapeutic range using a germination test for early growth is necessary as the toxicity threshold for Zn, as well as other micronutrients, is very sharp and exceeding this can cause deleterious effects to the plants. Raskar and Laware evaluated NP effects on onion seed germination and showed that the ZnO NP applied at low concentrations increases cell division, mitotic index and also germination, indicating that at low concentration ZnO NPs do not cause harm to the germination of seeds, and even improves these indices, [21] which was also observed in the present work for vigor, germination and root length.

Adhikari et al. also evaluated the effects of ZnO NP on growth of corn for a concentration of 50 mg Zn L<sup>-1</sup> and concluded that the application of ZnO NPs increased root length by 1.6 times when compared to the control [13]. This effect may be associated with nitrate reductase enzyme activity and increased antioxidant activity of plants [22]. Although, 50 mg L<sup>-1</sup> Zn NPs reduced the seed germination and root length of lettuce than control [23]. This reduction in growth might be due to experimental conditions and the increase in growth in current study [24]. Indicates that seed priming might be a suitable method to enhance plant growth with Zn supply

Despite that, many doubts still exist about the size cut off of NPs absorbed by plants and how this absorption is altered by the environment and by the roots themselves, since the entrance of the particle can be by means of insults, lenticels, simplastic or apoplastic or even by biotransformation. ZnO NP can adhere to the root surface root and enter in the plant cell. They also observed that ZnO NP was present in the apoplast and protoplast of the root, indicating that ZnO NP can be incorporated by plants [25].

In terms total fresh and dry biomass production, both the soluble source ZnCl<sub>2</sub> and ZnO NP promoted statistically significant increases with increasing concentration, so the results show the importance of Zn for growth and development since the total dry biomass production was lower in the

treatment devoid of zinc. Despite these effects, on average, application with ZnO NP showed higher dry biomass productions gains, as reported by other authors [7,13,26].

Some research has shown inhibitory or toxic effects for plants or crops in general. These effects only occur in extremely high ZnO NP concentrations of 400 to 2000 mg L<sup>-1</sup> [27]. On the other hand, lower doses of well-positioned and tested ZnO NPs may bring benefits to crops since the amount applied is lower, utilization efficiency is increased, nutrient loss is lower, and adverse impacts on the environment are smaller, when compared to larger size particles and soluble sources. These factors are important in the context of sustainable agriculture, since the development of plants can be satisfactory with a smaller amount of fertilizers increasing the efficiency of the applications that is only of 30–50% in average [27].

Plant nutrition with Zn plays a key role in germination, emergence of seedlings, establishment of population in addition to crop growth and development, and productivity [28]. These effects promoted by Zn are directly linked to the functions of this element in the plant protein synthesis, cell elongation, auxin biosynthesis and resistance to stresses [28]. Therefore, seedlings that emerge from seeds with low zinc availability will have less ability to establish in the environment, especially in soils with low levels of Zn. To remedy this deficiency inorganic zinc fertilizers sources are increasingly used, with a focus on evaluating the efficiency of different application forms of the insoluble ZnO source in nanometer scale for diverse cultures. Some research shows positive effects of the ZnO NP application for plants [1,10,14,29,30], although other authors report negative effects [31,32]. This duality of reported responses is mainly related to the amounts of ZnO NP applied, since there is a narrow line between the concentration that will promote beneficial effects and the concentration leading to a toxic effect for the plants. In addition, different plant species will respond to the same concentration differently, as well as the application form of these ZnO NP, which can also delivery via foliar, soil or seeds.

## 5. Conclusions

It is inferred, from the results of the current research, the hypothesis that ZnO NP can enhance seed vigor, germination and the development of corn seedlings when the NP~20 nm ZnO was applied through an 8 h seed priming in water. The optimal responses for the ZnO NP source varied between 70 and 90 mg L<sup>-1</sup> for the variables related to seed growth and germination. Furthermore, The ZnO NP source promoted beneficial effects when compared to control, while also being more efficient in improving seedling growth when compared to bulk ZnO and soluble Zn sources, since the concentrations to obtain the positive results were lower, especially for the biomass production which was also increased by ZnCl<sub>2</sub> but in a lower magnitude than for the ZnO NPs. Particle size plays an important role in the reactivity of NPs, and care must be taken to define what concentration to use, since excessive doses may inhibit seedling growth. Thus, the results presented here demonstrate that the Zn micronutrient can be supplied to corn seeds through a simple seed priming technique with ZnO NPs. Although, efforts are required to understand how NPs can act as fertilizer, since NPs seed priming is an incipient field and more studies are needed to spread a complete awareness about this NPs delivery system and micronutrient management.

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