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Zn Oxide Nanoparticles and Fine Particles: Synthesis, Characterization and Evaluation of the Toxic Effect on Germination and Vigour of *Solanum licopersicum* L.

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Abstract: The micronutrient Zinc (Zn) is essential for the proper growth and development of crops. Zinc oxide nanoparticles (NPs) and fine particles are presented as an emerging alternative to more traditional fertilizers. In this study, the possible toxic effect of four laboratory-synthesized ZnO NPs and fine particles with different characteristics on tomato seed germination and vigor parameters was examined in comparison to bulk ZnO. Different metal precursors were used for the chemical synthesis of the particles: $Zn(NO_3)_2$ and $ZnSO_4$, for particles named NIT-. and SUL-., respectively. In addition, the synthesis process was modified to obtain coated particles (denoted as UW-, NIT-UW and SUL-UW) and washed particles (denoted as W-, NIT-W and SUL-W). These particles were applied at different toxic doses (0, 1.4, 2.8, 5.6 and 11.2 g L^{-1}). The results indicated that although the constant contact time between the ZnO particles did not affect the germination capacity of the seeds, it affected the growth of roots and hypocotyls, with a higher impact on the roots' development. This toxicity was more evident from the lowest particle dose used, although it did not prevent radicle and hypocotyl elongation during the development period studied (14 days). The synthesized coated particles (NIT-UW, SUL-UW) generated high toxicity on radicle and hypocotyl development, and this effect was observed from the first days of contact with the particles. The observed toxic effects on radicle length were minimized by the application of bulk ZnO particles. In the case of hypocotyl growth, these minor toxic effects were observed by using NIT-W particles and bulk ZnO. The possibility of positive effects on seed germination and development (radicle and hypocotyl length) when in continuous contact with ZnO, whether in fine particles, nanoparticles, or bulk sizes, was excluded. Furthermore, no benefits on germination parameters were observed by suppressing the final washing step in the particle's synthesis process, suggesting that particle coating did not provide any advantage for seed germination under these continuous contact conditions.

Keywords: germination; bulk Zinc oxide; hypocotyl length; radicle length; tomato; Zinc oxide nanoparticles

1. Introduction

In plants, Zinc (Zn) serves as an essential trace element, playing a fundamental role in various physiological and metabolic processes [1,2]. The optimal concentration range of Zn for plants is narrow, emphasizing the importance of a precise dosage and controlled application. A suitable range of Zn concentrations in the plant is between 20 and 100 mg Zn kg^{-1} dry matter [3,4]. In tomatoes, Zn contents of around 30 mg Zn kg^{-1} (24.8 to 38.3 mg Zn kg^{-1} [5,6]) have been documented. However, concentrations vary according to the variety of tomato [7,8]. In this sense, the effects of Zn deficiency on plants have been reported by different authors [1,2,9]. These symptoms include chlorosis and smaller leaves, increasing crop maturity period, spikelet sterility and inferior quality of harvested products. These symptoms arise due to the disruption of essential functions like photosynthesis and protein synthesis, both reliant on proper Zn presence [9].

On the other hand, exceeding the critical Zn concentration within the plants can lead to toxicity symptoms, and the release of Zn into the environment has the potential to cause environmental issues [10], making it crucial to maintain proper management practices. Zn toxicity in plants may be manifested in various ways, including reduced yield, poor seed germination, stunted leaf growth and inhibited root development [11].

The availability of Zn in the soil varies widely [12], and low availability in soil (10–30 mg Zn kg^{-1}) can lead to Zn deficiency in plants, which negatively affects their growth [13]. Thus, the application of micronutrient sources like Zn has become increasingly significant over the past decade [14]. This is aimed at achieving appropriate Zn concentrations in the soil, which is crucial for optimal plant growth.

Nanotechnology has allowed for the development of nanoparticles (NPs) with unique properties that enable a wide range of applications depending on their size [15]. To classify a particle as a NP or ultrafine particle, its diameter must be between 1 and 100 nm. Particles larger than 100 nm and up to 2500 nm are classified as fine particles. Indeed, the use of Zn NPs as nanofertilizers has also aroused great interest. This interest lies in the fact that the characteristics of these particles are an advantage for supplying the desired Zn content within the plant, as they can be easily absorbed by the plant due to their small size [16]. However, there is a great concern about the potential adverse effects of Zn NPs on the environment and health. Its accumulation at high levels in the plant could not only affect plant growth but could also pose a route for contamination of the food chain [17]. It is also raising queries about their effects on vital processes such as seed germination [18].

Improving plant seed germination and seedling early developmental stages is one of the most notable applications of NPs in agriculture. Recent reports studied the impact of diverse NPs on various crops, particularly during early development, focusing on seed germination and seedling growth. Seed germination, involving radicle emergence and hypocotyl elongation, stands out as the most sensitive phase in a plant's life cycle. The use of NPs in seed coating aims to enhance seed performance beyond traditional techniques by improving germination rates, fostering leaf and root development, and bolstering resistance to environmental stress, pests and diseases [19]. The results of different studies have presented contrasting impacts, both positive and negative, of metal NPs on higher plants, predominantly agricultural species [20]. The plant's response to NPs presence is contingent upon several variables, including NP type, size, environmental concentration, time of exposure, plant variety and whether the NP serves as a vital micronutrient required by the plant, such as Cu, Zn, or Fe [21].

Regarding Zinc oxide (ZnO) NPs, there are different outcomes. Its phytotoxicity has been demonstrated as it inhibits root growth and development [22]. However, other studies showed that priming with ZnO NPs exhibits a detectable level of germination enhancement compared to the control seeds [23,24]. Another proven benefit of the application of ZnO NPs is their capacity to enhance the plant's antioxidant defense system and serve as an effective growth regulator, thereby improving crop productivity even under stressful conditions [25].

Critically, the germination resulting from seed priming is closely related to the concentrations of NPs used [26]. ZnO NPs can be used as nanofertilizers at optimal concentrations between 1 and 10 ppm since high concentrations can cause toxic effects, e.g., reduction in radicle and hypocotyl growth at concentrations above 50 ppm [27]. Furthermore, plants' responses to metal-containing NPs also depend on other factors, including the type of metal, plant species, exposure conditions and plant growth stage [28]. In tomato, authors have studied the effects of ZnO NPs application on seed germination, where low concentrations have a positive impact on the physiological profile and root architecture of tomato plants, improving their overall health and growth [29,30].

The general objective of this work is to investigate the effect of the application of different toxic doses of ZnO NPs, fine particles, or bulk-size particles with different characteristics and synthesized through different chemical methods, on the germination of a common agricultural crop, *Solanum licopersicum* L. Specific objectives include:

- Investigate the effect on the number of germinated seeds.
- Investigate the effect on radicle and hypocotyl length at different periods (7 and 14 days after germination).
- Investigate possible toxic effects in relation to the dose.

The hypothesis is that the physico-chemical characteristics of the different nanoparticles synthesised may influence their toxic effect at high doses.

2. Materials and Methods

2.1. Synthesis and Characterisation of ZnO Particles

The Zn sources used in this study were different ZnO fine particles and NPs synthesised in the laboratory as well as commercial bulk ZnO particles. Regarding the selection of the particle synthesis method, the method with the best production yield–cost ratio was chosen compared to other considered methods [31,32]. The procedure followed for the synthesis of the particles was based on the co-precipitation method described by Huy et al. (2019) [33] with the modification of the hydroxide source (NH_4OH) and metal precursors used. Different metal precursors, $Zn(NO_3)_2$ and $ZnSO_4$, were used for the synthesis of NIT-. and SUL-., respectively. In addition, the method was modified to obtain unwashed (denoted as UW-, NIT-UW and SUL-UW) and washed (denoted W-, NIT-W and SUL-W) particles since the by-products formed in the synthesis, namely NH_4NO_3 and $(NH_4)_2SO_4$, could have positive effects on seeds germination or plant growth, due to the presence of these primary (N) and/or secondary (S) macronutrients in the composition of the particles. In fact, they are known as common fertilizers. These by-products are originated by the combination of the Zn salt anion with the ammonium ion of the added base catalyst. Briefly, the synthesis process started by dropwise addition of 180 mL of 0.3 M NH_4OH solution into a glass beaker containing 180 mL of 0.15 M of the metal-containing precursor solution. Then, the solution was stirred vigorously for 180 min at room temperature, and the initially colourless solution turned milky-white after reaction, which indicates the formation of Zn-based particles compounds. The solution was divided into two portions, one portion was rinsed three times with distilled water (W) and the other was dried without be washed before (UW). Both samples were then dried at 80 °C for 24 h.

Hydrodynamic size and Zeta potential (ZP) measurements of the particles were recorded using dynamic light scattering (DLS) and electrophoretic migration techniques in a Zetasizer Nano ZS equipment (Malvern Panalytical Ltd., Malvern, UK). The particle size distribution was measured by transmission electron microscopy (TEM) using a JEOL JSM 6335F microscope and Image J 1.53e software. SEM images were obtained by using a JEOL JSM 6335F scanning electron microscope. The specific surface area and pore size distribution were obtained by using an N_2 adsorption volumetric system at 77 K (Micromeritics ASAP 2020). The amount of carbon, hydrogen, nitrogen and sulfur in the samples was analyzed using a LECO CHNS-932 elemental microanalyzer. Finally, the thermogravimetric analysis was accomplished in a Labsys Evo DSC/TGA Setaram thermogravimetric analyzer from 30 to 1200 °C.

2.2. Germination Tests

The influence of the synthesized particles (NIT-UW, NIT-W, SUL-UW, SUL-W) and bulk size ZnO particles (ZnO-bulk) on the germination of tomato seeds (*Solanum licopersicum* L.) was studied. For this purpose, different aqueous solutions of 10 mL with different toxic doses of Zn (0, 1.4, 2.8, 5.6 and 11.2 g L^{-1}) were prepared and subjected to a sonication process. In the control treatment, 10 mL of distilled water was added. The treatments were accomplished on the surface of a Petri dish filter paper. Taking into account the surface on which the solution was applied, these doses correspond to 0, 15, 30, 60 and 120 kg Zn ha^{-1}, respectively.

Subsequently, 20 tomato seeds were placed on the Petri dish, maintaining the required distance between them to allow for the elongation of the radicles and hypocotyl. Thus, Petri dishes were sealed with waxed paper to prevent moisture loss and placed in a culture cabinet under dark conditions and at 25 °C temperature. After 7 days, the number of germinated seeds was counted, and hypocotyls and radicle length were measured with a millimeter rule. These measurements were repeated after 14 days. It is noteworthy that the tests were conducted in randomized complete blocks, and 3 replicates of each treatment were conducted.

2.3. Data Analysis

For the analysis of the data obtained, dose–response curves were made, representing the relationship between the ZnO doses to which the seeds were exposed and the effect or response of the seeds to each dose. For the graphing of these curves, the values of EC_{10} (ZnO concentration that produces a 10% reduction in the response with respect to the control) or EC_{50} (ZnO concentration that produces a 50% reduction in the response with respect to the control) were used according to the results obtained. The experimental data were described by different models, establishing the least squares sum between observed and predicted data.

Different models were used depending on the behavior of the parameters and doses studied:

(i) Three-parameter logistic model [34], which considers Y_{max} (maximum response, i.e., the highest value of radicle or plumule height), EC_{10} and b (maximum slope of the model) as main variables;

$$Y(c) = \frac{Y_{max}}{1 + \frac{x}{100-x}\left(\frac{c}{EC_C}\right)^b}$$

(ii) Four-parameter logistic model [35,36], where Y_{min} (minimum response, when a minimum value is reached and maintained with several application rate values) is included as a variable.

$$Y = Y_{min}\frac{Y_{max} - Y_{min}}{1 + \left(\frac{x}{100-x}\right)\cdot\left(\frac{conc}{LCx}\right)^b}$$

The NOEC values (No Observed Effect Concentration), i.e., the maximum concentration at which no effect was observed, and LOEC (Lowest Observed Effect Concentration), i.e., the minimum concentration at which an effect was observed, were also determined. In addition, a Principal Component Analysis (PCA) was performed using the obtained particle characteristics and germination results.

Statistical analysis was accomplished by using Statgraphics Centurion XVII 17.2 software (Manugistic Inc., Rockville, MD, USA) and IBM SPSS Statistics 26.0 software (SPSS Inc., Chicago, IL, USA). ANOVA analyses were performed for comparison of mean values (Fisher LSD test at 95.0% confidence level). Also, NOEC and LOEC values were determined by Dunnett's mean separation analysis (significance level of 0.05). Finally, orthogonal analyses were also conducted to study possible differences between applying ZnO vs. the control treatment.

3. Results and Discussion

3.1. Characterization Results of ZnO Particles

The hydrodynamic diameter refers to the size of the hypothetical sphere that exhibits the same diffusion coefficient as the particle under measurement, considering the presence of a hydration layer around the particle or molecule. Consequently, the hydrodynamic diameter values provide an idea of the expected particle sizes. In our study, NIT-UW particles showed an average hydrodynamic diameter value of 603.3 nm, showing peaks with a mean value of 2380 nm in different registers, with an area between 16% and 33%. Therefore, the presence of some larger-sized particles contributes to the increase in the mean value. On the other hand, NIT-W particles exhibited homogeneous values, with an average hydrodynamic diameter of 541.0 nm. Samples SUL-UW and SUL-W were not analyzed due to their larger particle size distribution, inhibiting precise characterization by the DLS technique, as this method only performs optimally for the analysis of samples with a monodisperse distribution.

In our study, the particles exhibited sizes larger than those documented by previous researchers. According to Ganesan (2020) [37], an average hydrodynamic size of 338 nm was measured for the synthesized ZnO particles obtained biologically with an aqueous fungal extract. Concerning NIT-UW, its increased average value is attributed to the presence of a small quantity of larger particles, which notably impacted the overall hydrodynamic size value.

ZP values measured in deionized water of ZnO samples are shown in Table 1. ZP values are an indicator of the level of repulsion or attraction between particles. Thus, values ranging between −30 and 30 mV signify instability, suggesting that the solution in water is prone to forming unstable suspensions and aggregates. Since this value is the measure of the surface charge of the particle, the higher the ZP, the greater the repulsion between the particles, which can prevent agglomeration and, therefore, the higher the stability. In this case, as can be seen in Table 1, only NIT-W particles showed ZP values higher than 30 mV (30.6 ± 4.0 mV), indicating more stability in the water of these particles compared to the remaining samples. Other authors report highly variable ZP values with different synthesis methods [38–40].

Table 1. Main physico-chemical properties of ZnO particles.

	NIT-UW	NIT-W	SUL-UW	SUL-W
Zeta potential (mV)	6.3 ± 0.5	30.6 ± 4.0	−11.0 ± 3.7	−7.4 ± 5.7
%N	15.78 ± 1.97	0.26 ± 0.03	8.02 ± 0.32	0.54 ± 0.02
%C	0.15 ± 0.05	1.54 ± 0.20	0.07 ± 0.01	0.12 ± 0.02
%H	2.87 ± 0.18	0.71 ± 0.03	3.57 ± 0.09	2.38 ± 0.12
%S	0.03 ± 0.02	0.08 ± 0.10	14.90 ± 0.46	5.51 ± 0.13
S_{BET} (m^2 g^{-1})	-	20.0	0.4	9.2
V_{Mic} (cm^3 g^{-1})	9.5×10^{-5}	8.1×10^{-3}	1.2×10^{-4}	3.3×10^{-3}
V_{Meso} (cm^3 g^{-1})	-	3.1×10^{-2}	1.7×10^{-4}	1.1×10^{-3}
V_{Total} (cm^3 g^{-1})	5.0×10^{-6}	3.9×10^{-2}	2.9×10^{-4}	1.4×10^{-2}
V_{Mic}/V_{Total}	-	0.21	0.41	0.24
Average pores size (nm)	-	7.8	3.3	6.0

S_{BET}: Specific surface area, obtained by the Brunauer, Emmett and Teller theory; V_{Mic}: micropores volume; V_{Meso}: mesopores volume; V_{Total}: total pores volume.

SEM technique was used to capture in-depth micrographs of the synthesized samples, mainly to reveal their morphology and estimate their approximate particle size. Samples NIT-UW and NIT-W exhibited particle sizes larger than expected, with estimated values of 200 and 300 nm, respectively (Figure 1). On the other hand, SUL-UW and SUL-W samples presented a lamellar structure with an estimated thickness layer below 50 nm. The layers in the SUL-W sample exhibited smaller sizes, likely due to breakage occurring during the washing process or removal of the salt by-product coating. For NIT-UW and SUL-UW samples, which were not washed after synthesis, a layer covering the particles was visible in SEM micrographs, like a gluing phase. This layer most likely consisted of salt by-products, as already referred to. Additionally, TEM analysis was conducted for the washed samples (NIT-W and SUL-W), obtaining particle size values of 91 and 104 nm, respectively. In addition, a higher aggregation of the samples NIT-UW, SUL-UW and SUL-W was observed.

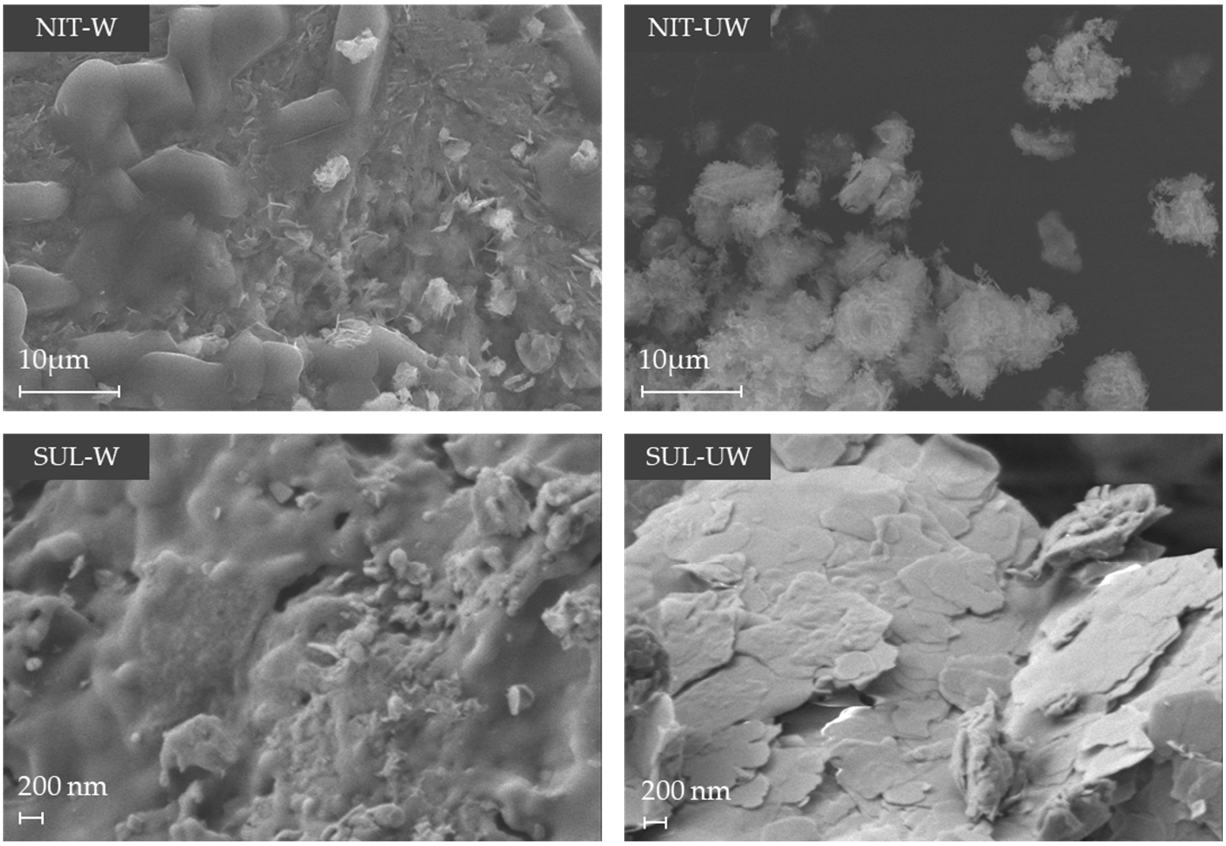

Figure 1. SEM micrographs of the synthesized ZnO NPs.

Nitrogen (N), carbon (C), hydrogen (H) and sulfur (S) contents in the synthesized samples can be seen in Table 1. The general amounts of N and S agree with the presence of NH_4NO_3 and $(NH_4)_2SO_4$ in the unwashed/washed samples of NIT-. and SUL-., respectively.

Moreover, different specific surface area values (S_{BET}) were obtained for the samples analyzed (Table 1). Samples that showed higher specific surface area values were NIT-W with 20.0 m^2 g^{-1}, followed by SUL-W sample, with a value of 9.2 m^2 g^{-1}, proving that the washing step removes the salt by-products that are filling the pores, which is consistent with the pore's enlargement seen in the table. The samples showing high S_{BET} values denote a high reactivity/interaction with other compounds, being directly related to the particle size and/or morphology of the material. Therefore, NIT-UW and SUL-UW particles showed very low or no detectable S_{BET} values. These results are directly related to the synthesis process of NIT-UW and SUL-UW samples since they are not washed after treatment, leading to a coating on the sample's surface (as can be seen in SEM micrographs), reducing the porous structure of the materials. The specific surface area measured for NIT-W NP (S_{BET} = 20.0 m^2 g^{-1}) is in agreement with the results obtained by Sharma and Ghose (2015) [41], ranging from 19.3 to 21.4 m^2 g^{-1}, and by Huy et al. (2019) [33] (S_{BET} = 20.02 m^2 g^{-1}), with the latter having a similar process of synthesis to the one used in this work.

Regarding the pores size values, all the samples analyzed showed much lower values than those reported by Huy et al. (2019) [33], 17.0 nm. Sample NIT-W shows the highest value of pore size, certainly due to a high removal efficiency of NH_4NO_3. On the other hand, the elemental analysis showed that sample SUL-W still presents some amount of

S element, explained by the lower solubility of $(NH_4)_2SO_4$ in water when compared to NH_4NO_3 (70.6 and 118 g/100 mL at 0 °C [42], respectively), or the presence of other S-containing species. Regarding the pore volume, the same conclusions can be drawn, and from the volume ratio of micro and mesopores, it can be concluded that the salt by-products mainly fill the mesopores.

The thermogravimetric curves measured for the synthesized ZnO particles are shown in Figure 2. The total weight loss of the NIT-W sample (Figure 2b), heated up to 1200 °C, was only 6%, showing a progressive mass loss between ~200 and 1200 °C. This behavior is indicative of the high thermal stability of NIT-W NPs due to their purer form. On the other hand, the other samples showed higher total mass loss values, e.g., 66, 68 and 40% for NIT-UW, SUL-UW and SUL-W particles, respectively. These higher weight losses are related to the thermal decomposition of the salt by-products. This result again agrees with the incomplete salt removal in sample SUL-W.

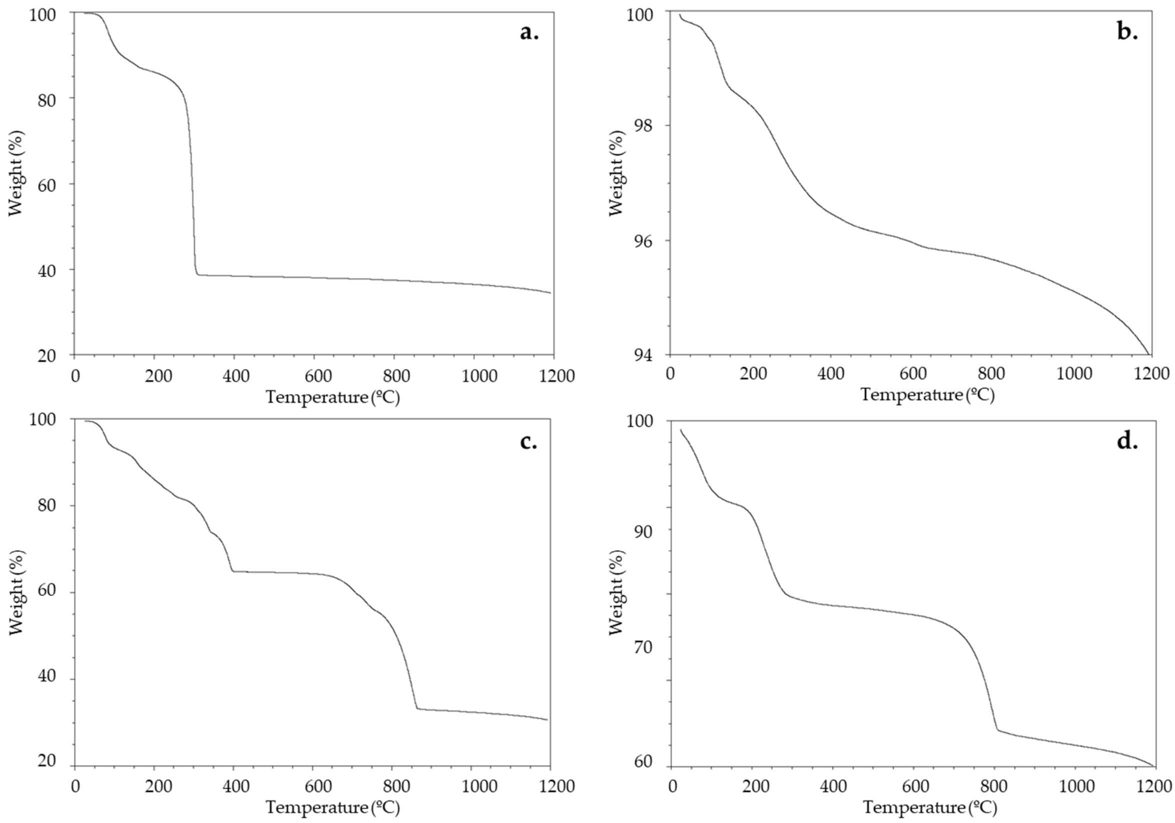

Figure 2. TGA curves: (**a**) NIT-UW sample; (**b**) NIT-W sample; (**c**) SUL-UW sample; (**d**) SUL-W sample.

In all cases, the mass loss observed between 30 and 150 °C (18, 1.5, 10 and 10% for NIT-UW, NIT-W, SUL-UW and SUL-W samples, respectively) can be attributed to the evaporation of water generally occluded in the internal structure of the particles. This indicated the hydration of the ZnO powder. Likewise, mass loss observed at temperatures ranging from 160 to 450 °C (47 and 27%, for NIT-UW and SUL-UW, respectively) can be attributed to the decomposition of the saline by-products used in the synthesis of the particles (NH_4NO_3 for NIT-. and $(NH_4)_2SO_4$ for SUL-.). In the decomposition of SUL-UW, two stages were observed, mainly attributable to the thermal decomposition of ammonium nitrate, as shown in previous studies [43]. The mass losses observed between 600 and 800 °C are due to incomplete conversion of $ZnSO_4$ since the thermal degradation of this salt is consistent with this range of temperatures [44].

At temperatures above 1000 °C, generally, there is no mass loss since Zn metal is stable at that temperature. In addition, no weight losses were observed at temperatures above

400 °C in NIT-UW and 900 °C in SUL-UW and SUL-W samples, confirming the completion of the thermal decomposition reaction and the formation of ZnO particles. However, it was observed that NIT-W and SUL-W samples were not stable above temperatures of 800–850 °C. So, it was observed that in the coated samples (NIT-UW and SUL-UW), nitrate and sulfate salts remained in the sample at higher amounts. Furthermore, for NIT-W and SUL-W particles, it was observed that even after the washing process, the salts remained in the samples, although in lower amounts.

3.2. Influence of ZnO Particles on Germination Parameters

3.2.1. Seed Germination Percentage

The contact effect of different sources and application doses of Zn on the germination grade of *Solanum licopersicum* L. seeds is shown in Table 2.

Table 2. Contact effect of different Zn doses on *Solanum licopersicum* L. seeds germination percentage.

	NIT-UW		NIT-W		SUL-UW		SUL-W		ZnO-bulk		
Dose ($g\ Zn\ L^{-1}$)	**Mean ± SD (%)**		**Mean ± SD (%)**		**Mean ± SD (%)**		**Mean ± SD (%)**		**Mean ± SD (%)**		***p*-Value [1]**
0	95.0 ± 5.0	b	95.0 ± 5.0	-	95.0 ± 5.0	-	95.0 ± 5.0	-	95.0 ± 5.0	-	
1.4	86.5 ± 3.0	b	86.5 ± 6.0	-	88.5 ± 10.5	-	83.5 ± 6.0	-	81.5 ± 14.5	-	*NS*
2.8	93.5 ± 3.0	b	93.5 ± 3.0	-	85.0 ± 5.0	-	91.5 ± 7.5	-	85.0 ± 8.5	-	*NS*
5.7	90.0 ± 5.0	b	80.0 ± 8.5	-	86.5 ± 3.0	-	88.5 ± 7.5	-	86.5 ± 12.5	-	*NS*
11.4	56.5 ± 27.5	a	81.5 ± 20.0	-	86.5 ± 10.5	-	88.5 ± 3.0	-	86.5 ± 7.5	-	*NS*
p-value [2]	0.024		*NS*		*NS*		*NS*		*NS*		
Orthogonal contrasts [3]	*NS*		*NS*		*NS*		*NS*		*NS*		

All values compared using the LSD multiple range test at the 0.05 probability level. Statistical differences are presented with different letters (lowercase letters indicate differences between doses—for the same source—and uppercase letters indicate differences between sources—for the same dose). *NS: not significant*. [1] *p*-values obtained between the different ZnO sources for each dose. [2] *p*-values obtained between the different doses for each source of Zn. [3] Orthogonal contrasts control vs. Zn dose.

The different treatments exhibited maximum reductions in germination percentage of 40.4, 15.8, 10.5, 12.3 and 14.0% when compared to the control, for NIT-UW (11.2 $g\ Zn\ L^{-1}$ dose), NIT-W (5.6 $g\ Zn\ L^{-1}$ dose), SUL-UW (2.8 $g\ Zn\ L^{-1}$ dose), SUL-W (1.4 $g\ Zn\ L^{-1}$ dose) and ZnO-bulk (1.4 $g\ Zn\ L^{-1}$ dose), respectively. However, the mean separation tests only revealed significant differences in germination when the highest dose of the NIT-UW sample (11.2 $g\ Zn\ L^{-1}$) was used. At this dose, the mean percentage of germinated seeds was significantly lower (56.5%). However, it should be noted that the results in this case show high variance.

No significant differences among the different particles for each dose were observed in all cases. The orthogonal contrasts, used to compare the effect of the ZnO treatments vs. the control group (without ZnO), also indicated no variations in the mean percentage of germinated seeds. As has been reported in the literature, plants require moderate levels of Zn to grow and develop properly [12]. According to other authors, in the case of tomato, treatments with ZnO NPs at low doses show no significant differences in the seed germination percentage in relation to the control [45], even improving the percentage [30]. However, at higher concentrations of ZnO NPs, a decrease in seed germination percentage is observed in tomato plants [26,29]. Germination depends not only on the amount of ZnO used in the treatment but also on the type of NPs. According to Sánchez-Pérez et al., 2023 [46], treatment with ZnO NPs obtained via green synthesis improved seed germination rate, while chemically synthesized ZnO NPs at higher concentrations decreased germination percentage.

In the case of the treatment with NIT-UW particles, the obtained NOEC and LOEC values corresponded to concentrations of 5.6 and 11.2 $g\ Zn\ L^{-1}$, respectively. These particles are characterized by a high N content (15.78%) by the presence of NH_4NO_3 salt residues and undetectable specific surface area values (not/very low porous structure).

For the rest of the treatments, these values cannot be determined as no statistically significant reduction in the number of germinated seeds was measured. These results suggested low contact toxicity of ZnO on tomato (*Solanum licopersicum* L.) seed germination. Only the highest dose (11.2 g Zn L^{-1}) of NIT-UW particles led to a significant reduction in the number of germinated seeds.

3.2.2. Radicle Length

The contact effects on the elongation of the radicle exhibited by the seeds at 7 and 14 days of development, according to the dose and sources of Zn applied, are shown in Table 3. These best-fitted models were the 3-parameter logistic model [34] for NIT-UW and SUL-UW samples and the 4-parameter logistic model [35,36] for NIT-W, SUL-W and ZnO-bulk. The obtained fitting parameters can be seen in Table 4.

Table 3. Contact effects of different ZnO treatments on mean radicle length of tomato seeds at 7 and 14 days of development.

7 Days											
	NIT-UW		**NIT-W**		**SUL-UW**		**SUL-W**		**ZnO-Bulk**		
Dose (g Zn L^{-1})	**Mean± SD(mm)**		**Mean± SD(mm)**		**Mean± SD(mm)**		**Mean± SD(mm)**		**Mean± SD(mm)**		***p*-Value [1]**
0	80.23 ± 17.70	b	80.23 ± 17.70	b	80.23 ± 17.70	b	80.23 ± 17.70	b	80.23 ± 17.70	b	
1.4	6.81 ± 1.48	aAB	8.84 ± 2.11	aCD	4.46 ± 0.62	aA	6.89 ± 1.49	aAB	10.28 ± 0.47	aD	0.0042
2.8	3.17 ± 0.44	aA	8.35 ± 2.09	aB	2.95 ± 0.131	aA	2.82 ± 0.32	aA	9.92 ± 3.89	aB	0.0025
5.6	2.25 ± 0.46	aA	9.46 ± 1.79	aB	2.68 ± 0.482	aA	3.52 ± 1.49	aA	12.65 ± 3.28	aC	0.0001
11.2	1.37 ± 0.33	aA	8.19 ± 1.10	aC	1.40 ± 0.373	aA	3.00 ± 0.35	aB	7.63 ± 0.94	aC	0.0000
p-value [2]	0.0000		0.0000		0.0000		0.0000		0.0000		
14 Days											
	NIT-UW		**NIT-W**		**SUL-UW**		**SUL-W**		**ZnO-Bulk**		
Dose (g Zn L^{-1})	**Mean± SD(mm)**		**Mean± SD(mm)**		**Mean± SD(mm)**		**Mean± SD(mm)**		**Mean± SD(mm)**		***p*-Value [1]**
0	80.75 ± 3.99	c	80.75 ± 3.99	b	80.75 ± 3.99	b	80.75 ± 3.99	c	80.75 ± 3.99	c	
1.4	7.42 ± 2.59	bB	9.13 ± 1.93	aBC	4.51 ± 0.37	aA	6.93 ± 1.36	bAB	11.41 ± 1.47	abC	0.0024
2.8	3.24 ± 0.73	aA	9.01 ± 0.77	aB	3.95 ± 0.81	aA	3.91 ± 1.00	abA	10.33 ± 3.85	abB	0.0037
5.6	2.31 ± 0.31	aA	9.88 ± 2.20	aB	2.70 ± 0.44	aA	3.74 ± 0.53	abA	13.39 ± 3.48	bC	0.0001
11.2	1.42 ± 0.44	aA	8.93 ± 1.51	aB	2.37 ± 1.44	aA	3.12 ± 0.53	aA	7.68 ± 0.86	aB	0.0000
p-value [2]	0.0000		0.0000		0.0000		0.0000		0.0000		
Elongation between 7 and 14 Days (mm)											
Dose (g Zn L^{-1})	**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		
1.4	0.06		0.41		0.02		0.22		0.75		
2.8	0.06		0.66		1.00 *		1.09		0.41		
5.6	0.61		0.29		0.05		0.03		1.14		
11.2	0.73		0.97		0.12		0.05		0.06		

All values compared using the LSD multiple range test at the 0.05 probability level. Statistical differences are presented with different letters (lowercase letters indicate differences between doses—for the same source and development days—and upper-case letters indicate differences between sources—for the same dose and development days). [1] *p*-values obtained between the different ZnO sources for each dose and development days. [2] *p*-values obtained between the different doses for each source of ZnO and development days. * Significant differences between the mean elongation values from day 7 to 14.

Table 4. Model parameters for radicle length after 7 and 14 days of treatment.

		NIT-UW	NIT-W	SUL-UW	SUL-W	ZnO-Bulk
Model		3-Parameter	4-Parameter	3-Parameter	4-Parameter	4-Parameter
7 days	Y_{min}	-	8.67	-	3.11	10.07
	Y_{max}	80.23	80.23	80.23	80.23	80.23
	EC_{10}	1.04	10.66	0.05	12.77	11.99
	b	0.9	17.69	0.50	18.42	25.96
	TSS	633.6	656.3	629.2	633.9	718.6
14 days	Y_{min}	-	66.90	-	21.30	61.35
	Y_{max}	89.00	89.27	89.31	89.28	89.10
	EC_{10}	3.88	13.66	2.01	7.61	7.58
	b	1.15	23.71	1.00	3.65	2.99
	TSS	314.6	762.1	152.4	166.1	2631.3

Y_{max}: maximum response (maximum radicle length achieved with the model); Y_{min}: minimum response (minimum radicle length obtained by the model); EC_{10}: ZnO concentration that generates a 10% reduction with respect to the maximum effect; b: maximum slope of the model; TSS: total sum of squares (the model that provided the minimum TSS was chosen).

In general, a similar trend in radicle growth was observed at both stages of development studied (7 and 14 days). The LOEC and NOEC values (1.4 and 0 g Zn L^{-1}, respectively) indicated a negative effect on radicle elongation at very low concentrations. So, these results suggested that the sources are highly toxic to radicle development. According to other authors [26,46], increasing the dose of ZnO NPs decreases radicle length with respect to the control.

However, at 14 days, the results obtained showed a higher influence on the application dose of seed growth than those observed at 7 days. The results show that after 7 days of contact, an increase in dose from 1.4 g Zn L^{-1} did not lead to a significant decrease in radicle length. On the other hand, radicle length at 14 days shows a significant decrease at 2.8 g Zn L^{-1} (96.0% at NIT-UW-30 and 95.2% at SUL-W-30, with respect to control), with respect to the same treatments given at a 1.4 g Zn L^{-1} dose (90.8% and 91.4%, respectively).

NIT-UW and SUL-UW particles showed the lowest radicle length values (reaching reductions of up to 98.3 and 98.2% for NIT-UW-120 at 7 and 14 days, respectively, and up to 98.3 and 97.1%, for SUL-UW-120 at 7 and 14 days, respectively). These particles were not washed, so the N composition is high (15.78 and 8.02% in NIT-UW and SUL-UW samples, respectively) when compared to the washed particles due to their coating consisting of NH_4NO_3, $(NH_4)_2SO_4$ and $ZnSO_4$ salts, respectively. The unwashed particles (NIT-UW and SUL-UW) demonstrated a contrast compared to the remainder of the particles, as their values adapted to the three-parameter model. The obtained EC_{10} values (Table 3) indicated higher toxicity on radicle length by using NIT-UW and SUL-UW particles at lower contact times. It should be noted that the lower EC_{10} values at 7 days compared to 14 days for NIT-UW, NIT-W and SUL-UW particles could indicate that the toxic effect occurs in the first days of seed contact with the particle, contrary to the behavior observed for SUL-W and ZnO-bulk particles.

In addition, it was observed that for each of the tested doses, radicle length was higher for treatment with the NIT-W sample compared to NIT-UW. This indicates a lower toxic effect of the washed NIT-W particles versus the unwashed (coated) NIT-UW sample. Despite both solids being synthesized following the same synthesis method, using $Zn(NO_3)_2$ as a precursor, the determined characteristics are very different. Thus, in general terms, SUL-W particles showed similar behavior to their unwashed counterparts (SUL-UW).

The application of the ZnO-bulk treatment resulted in higher radicle length compared to the synthesized particles for all doses and contact times tested (reduction for 1.4, 2.8, 5.6 and 11.2 g Zn L^{-1} doses after 7 days: 87.2, 87.6, 84.2 and 90.5%, respectively; after 14 days: 85.9, 87.2, 83.4 and 90.5%, respectively). Although other studies concluded that high doses of ZnO can cause inhibitory effects [27,47], our results indicated that regardless of the dose applied and the characteristics of the particles, the toxic effects on the radicle length of the synthesized particles were higher than those observed for the bulk-sized commercial particles. In the study conducted by Liu et al. (2016) [48], they obtained slightly higher inhibitory effects on root development when Zn ions ($ZnSO_4$) or ZnO NPs (average diameter of 36 ± 9 nm) were used versus ZnO-bulk particles. In this case, these authors suggested that the effects of Zn NPs were closely related to the enhanced release of Zn ions from the NP surfaces.

3.2.3. Hypocotyl Length

The contact effect exhibited by the seeds after 7 and 14 development days on the elongation of the hypocotyl changing the dose and sources of Zn used are shown in Table 5. These best-fitted three-parameter and four-parameter logistic models and the corresponding parameters can be seen in Table 6.

Table 5. Contact effects of different ZnO treatments on mean hypocotyl length of tomato seeds at 7 and 14 days of development.

7 Days											
	NIT-UW		**NIT-W**		**SUL-UW**		**SUL-W**		**ZnO-Bulk**		
Dose (g Zn L^{-1})	**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		***p*-Value [1]**
0	76.71 ± 7.49	e	76.71 ± 7.49	b	76.71 ± 7.49	c	76.71 ± 7.49	c	76.71 ± 7.49	b	
1.4	40.28 ± 2.43	dB	47.33 ± 1.25	aC	26.84 ± 3.45	bA	31.44 ± 4.06	bA	46.54 ± 5.24	aBC	0.0001
2.8	31.69 ± 4.85	cBC	41.45 ± 4.98	aCD	20.59 ± 3.09	bAB	13.98 ± 5.13	aA	47.31 ± 13.46	aD	0.0011
5.6	16.61 ± 2.56	bA	47.12 ± 5.31	aB	12.68 ± 3.34	aA	16.90 ± 1.24	aA	34.76 ± 16.35	aB	0.0014
11.2	6.58 ± 0.56	aA	49.01 ± 2.46	aD	6.72 ± 1.91	aA	13.84 ± 1.78	aB	42.17 ± 2.25	aC	0.0000
p-value [2]	0.0000		0.0000		0.0000		0.0000		0.0055		
14 Days											
	NIT-UW		**NIT-W**		**SUL-UW**		**SUL-W**		**ZnO-Bulk**		
Dose (g Zn L^{-1})	**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		***p*-Value [1]**
0	89.27 ± 5.08	e	89.27 ± 5.08	c	89.27 ± 5.08	e	89.27 ± 5.08	c	89.27 ± 5.08	b	
1.4	56.67 ± 6.74	dA	77.94 ± 4.73	bcB	49.43 ± 3.37	dA	50.57 ± 4.89	bA	75.49 ± 4.56	bB	0.0001
2.8	42.51 ± 5.89	cB	61.81 ± 8.76	aC	32.01 ± 3.39	cAB	24.82 ± 4.47	aA	68.76 ± 11.94	abC	0.0001
5.6	25.77 ± 1.63	bAB	67.70 ± 13.14	abC	22.40 ± 3.71	bA	23.71 ± 1.32	aA	47.67 ± 24.47	aBC	0.0047
11.2	10.72 ± 5.55	aA	71.19 ± 4.02	abC	11.04 ± 2.09	aA	19.53 ± 0.29	aB	72.39 ± 4.72	bC	0.0000
p-value [2]	0.0000		0.0148		0.0000		0.0000		0.0301		
Elongation between 7 and 14 Days (mm)											
Dose (g Zn L^{-1})	**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		**Mean (mm)**		
0	4.14		22.18 *		4.32		5.70		30.23 *		
1.4	9.17 *		20.58		9.72 *		6.81 *		12.91		
2.8	10.82		20.35 *		11.41 *		10.83 *		21.45		
5.6	16.39 *		30.62 *		22.59 *		19.14 *		28.95 *		

All values compared using the LSD multiple range test at the 0.05 probability level. Statistical differences are presented with different letters (lowercase letters indicate differences between doses—for the same source and development days—and uppercase letters indicate differences between sources—for the same dose and development days-). [1] *p*-values obtained between the different Zn sources for each dose and development days. [2] *p*-values obtained between the different doses for each source of Zn and development days. * Significant differences between the mean elongation values from days 7 to 14.

Table 6. Model parameters for hypocotyl length after 7- and 14-day treatments.

		NIT-UW	NIT-W	SUL-UW	SUL-W	ZnO-Bulk
Model		**3-Parameter**	**4-Parameter**	**3-Parameter**	**4-Parameter**	**4-Parameter**
7 days	Y_{min}	-	46.23	-	14.91	38.06
	Y_{max}	76.49	76.71	76.68	76.71	76.69
	EC_{10}	2.37	4.39	0.41	12.93	0.71
	b	1.08	55.75	0.77	21.55	1.09
	TSS	248.3	331.2	197.8	225.1	1256.8
14 days	Y_{min}	-	66.90	-	21.30	61.35
	Y_{max}	89.00	89.27	89.31	89.28	89.10
	EC_{10}	3.88	13.66	2.01	7.61	7.58
	b	1.15	23.71	1.00	3.65	2.99
	TSS	314.6	762.1	152.4	166.1	2631.3

Y_{max}: maximum response; Y_{min}: minimum response; EC_{10}: ZnO concentration that produces a 10% reduction with respect to the maximum effect; b: maximum slope of the model; TSS: total sum of squares.

All treatments showed a reduction in hypocotyl elongation compared to the control experiment. The LOEC and NOEC values (1.4 and 0 g Zn L^{-1}, respectively) indicated a negative effect on hypocotyl length at very low concentrations, as seen with radicle length.

The results at 14 days showed similar behavior to those obtained at 7 days, except for sources NIT-W and ZnO-bulk, which showed a significant increase in hypocotyl length between days 7 and 14, achieving a decrease with respect to the control of 12.7 and 15.4% for the dose of 1.4 g Zn L^{-1}, respectively.

Again, NIT-UW and SUL-UW particles showed the lowest values of hypocotyl length. Also, the model parameters showed a different behavior for the unwashed particles (NIT-UW and SUL-UW) with respect to the rest, as the model does not consider a minimum value of hypocotyl length. The obtained EC_{10} values (Table 5) corroborated the previous results obtained for radicle length, which indicated higher toxicity on seed development using NIT-UW and SUL-UW particles at lower contact times. These results suggested that the toxic effects occurred from the first days of seed contact with the particle. Elongation values indicate that although the toxicity of the sources leads to a decrease in hypocotyl length compared to the control, hypocotyls elongate as incubation time progresses (Table 5).

The mean separation test revealed significant differences in hypocotyl length between the application doses of the NIT-UW source. When the dose of NIT-UW was increased, a significant decrease in hypocotyl length was shown. This effect was observed both at 7 and 14 days after germination (decreases of up to 91.4% and 88.0% with 11.2 g Zn L^{-1} dose, respectively). However, the toxic effect of the washed homologous particle NIT-W was lower than when unwashed. The results at 7 days of NIT-W indicate that although there is a toxic effect of Zn contact on the seed, this effect does not increase with higher application doses.

In the same way, hypocotyl elongation of seeds in contact with SUL-UW particles showed significant differences when lower (1.4 and 2.8 g Zn L^{-1}) and higher doses (5.6 and 11.2 g Zn L^{-1}) were used at 7 days after germination. However, at 14 days, a clear difference in the dose effect was observed, showing greater toxicity with the increase in the contact dose.

The highest hypocotyl lengths were observed with sources NIT-W and ZnO in bulk size, being these the least toxic treatments for all the tested doses. Among the particles selected for this study, the NIT-W particles are the only ones that did not show a tendency to aggregate due to their high stability, as indicated by the ZP value (30.6 mV). The results obtained by DLS corroborate that the rest of the particles showed aggregation. Therefore, it can be considered that this sample maintained a constant particle size during the studies, and the rest of the particles may have undergone aggregation over the time elapsed between synthesis and characterization, displaying particle size values higher than those shown after treatments. As a result, these particles may have a much smaller size during the study owing to the sonication process they undergo before being added to the petri dishes. This phenomenon explains the increased toxicity of the smaller particles during the study (NIT-UW, SUL-UW and SUL-W).

The general symptom of Zn^{2+} phytotoxicity is a retardation of growth, with the plants being stunted [49]. Studies that investigate the seed exposure to treatments through limited exposure (priming, etc.), like the study conducted by Bayat et al. (2022) [28], suggest that NPs are absorbed on the surface of the seeds and are gradually re-released to exhibit their effects in the subsequent days. In our study, however, seeds underwent continuous exposure to treatment, showing a decrease in both radicle and hypocotyl growth.

In order to study the relationship between the characteristics of the synthesized ZnO particles and the toxicity behavior observed in the tested germination parameters, a Principal Component Analysis (PCA) was carried out (Figure 3). In the bi-plot graph of the two parameters, it was possible to distinguish different groups of values depending on the particle type and/or the used ZnO particle dose, indicating different behavior of the particles. This distribution shows differences between NIT-UW and SUL-UW (both unwashed particles), NIT-W (particles synthesized with $Zn(NO_3)_2$ precursor), and SUL-W (particles synthesized using $ZnSO_4$ precursor). It is observed that the behavior of NIT-UW particles (unwashed solids synthesized with $Zn(NO_3)_2$ precursor) was mainly influenced by their mass losses at 30–150 and 60–450 °C, as well as by the percentage of N content. However, the behavior of SUL-UW particles (unwashed particles synthesized using $ZnSO_4$ as a precursor) was mainly influenced by their H and S content. Particles SUL-W (synthesized from the precursor $ZnSO_4$) showed similar behavior, with the difference in the dose used (1.4 g Zn L^{-1}). These particles did not show a high influence on any of the studied parameters. Furthermore, NIT-W samples (particles synthesized from the precursor $Zn(NO_3)_2$) were mainly influenced by ZP, V_{Total} and $V_{Mic,}$ as well as the C content. It is noteworthy to highlight that NIT-W particles were also influenced by V_{meso}, S_{BET} and average pore size parameters (Figure 3B).

The PCA biplot graphs showed that, on the one hand, the parameters V_{Total} and V_{Mic} and, on the other hand, V_{Meso}, S_{BET} and mean pore size seem to be related to each other. This suggests that these parameters had similar behavior in the different synthesized particles under the experimental conditions tested.

The PCA biplot graph (Figure 3A) shows how the different characteristics of the synthesized ZnO particles influenced the tested germination parameters and, thus, the toxicity effect on the seeds. It is observed that the length of the seedling hypocotyl was positively correlated with the ZP and C content. The NIT-W particles caused a lower negative impact on hypocotyl length. These particles showed a high ZP value and high stability (ZP values ranging between −30 and 30 mV signify instability) and the highest percentage of C in their composition (1.54%). Furthermore, the distribution of the data indicates that the parameters V_{Total}, V_{Mic}, V_{Meso}, C content (Figure 3A), S_{BET} and pore size influenced radicle length (Figure 3B). NIT-W particles showed high values of V_{Total}, V_{Mic}, V_{Meso}, S_{BET} and pore size compared to unwashed particles. In addition, NIT-W stood out for its high C content. This particle showed a lower toxicity effect on radicle length. In contrast, these parameters determined from the particles did not seem to be related to germination percentage.

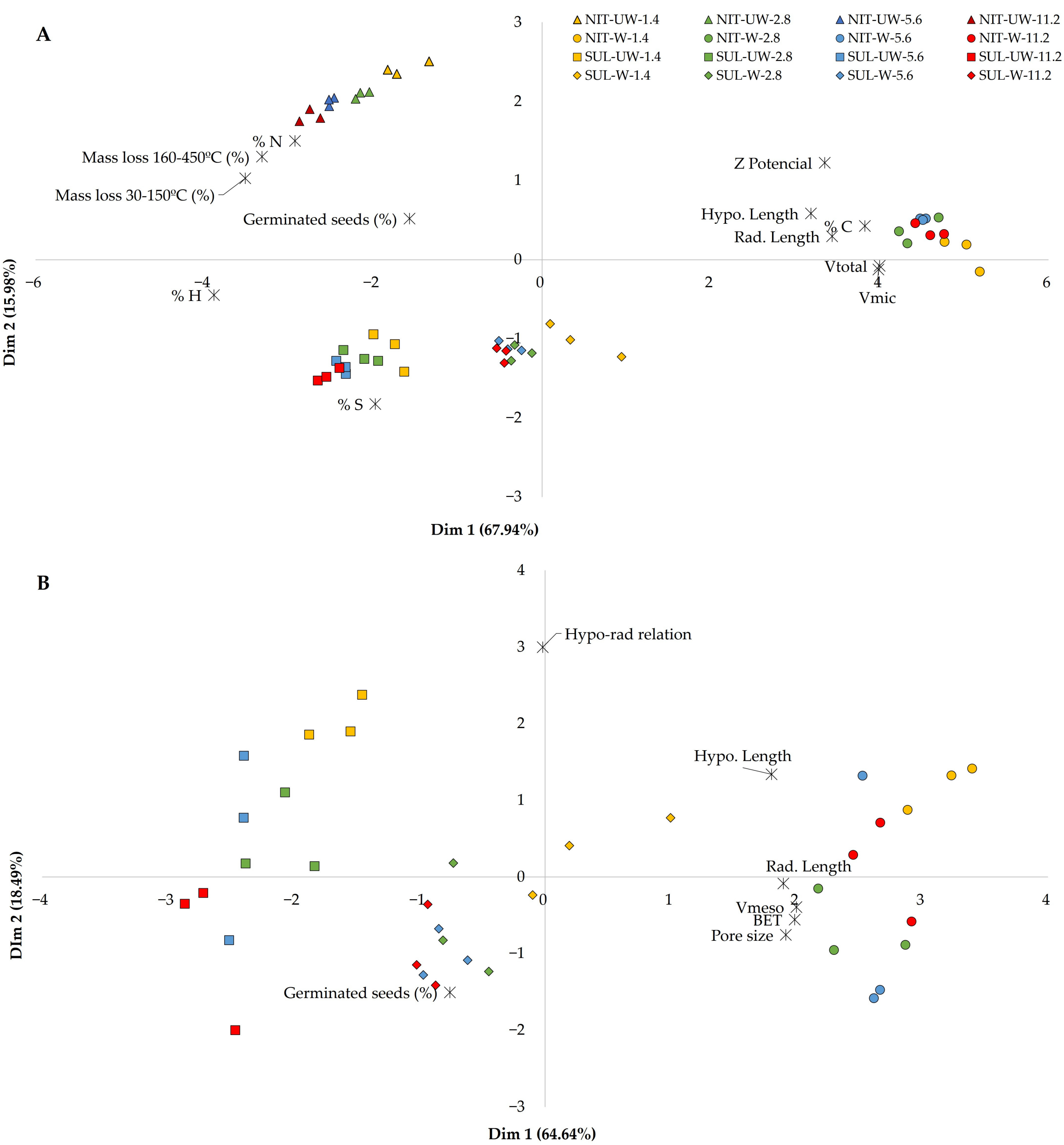

Figure 3. Bi-plot graph of the two principal parameters in the Principal Component Analysis (PCA) of the germination parameters studied (percentage of germinated seeds, radicle and hypocotyl lengths) and characteristics of the particles used: (**A**) Zeta potential; %N; %C; %H; %S; V_{Mic} (micropores volume); V_{Total} (total pores volume); mass loss at 30–150 °C, and at 160–450 °C; (**B**) S_{BET} (specific surface area); V_{Meso} (mesopores volume); pore size. The variance values measured for the 2 components were 89.92% (**A**) and 83.12% (**B**).

4. Conclusions

The findings of this study indicated that, although the continuous contact of the different ZnO particles did not show adverse effects on the germination capacity of tomato seeds, it negatively affected the development of roots and hypocotyls. These toxic effects were observed even at the lowest application dose of the study, indicating that it was an excessive dose, and were more pronounced on root development than on hypocotyl development.

The physical and chemical characteristics of the different nanoparticles had an impact on their toxic effect on seedling development. The radicle growth of tomato seedlings was affected when exposed to ZnO particles, and this effect depended on the particle properties, such as their Zeta potential (ZP), carbon content (C), specific surface area (S_{BET}), micropore, mesopore and total volume (V_{Mic}, $V_{Meso,}$ V_{Total} and size of the pores. Particles with high values of those properties, such as NIT-W, had a less harmful impact on tomato radicle length compared to other treatments studied. Moreover, regarding hypocotyl growth, its length was positively correlated with the ZP and C content of the synthesized particles applied. Particles with high values of ZP and C content, such as NIT-W, resulted in a less pronounced negative impact on tomato hypocotyl length.

Therefore, the possibility of positive effects on seed germination and seedling development (root and hypocotyl length) under continuous contact with ZnO particles, either in fine particles, nanoparticles or bulk size, was ruled out. In addition, the suppression of the final washing in the synthesis process of the synthesized particles has not been beneficial for the germination parameters studied in any of the synthesized particles, so it can be concluded that the existence of the by-products coating on the particles did not lead to any advantage regarding the germination of the seeds at these continuous contact conditions.

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