Comparison of the effects of commercial coated and uncoated ZnO nanomaterials and Zn compounds in kidney bean (*Phaseolus vulgaris*) plants

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Abstract

Bean (*Phaseolus vulgaris*) plants were grown for 45 days in soil amended with either uncoated (Z-COTE[®]) and coated (Z-COTE-HP1[®]) ZnO nanomaterials (NMs), bulk ZnO and ZnCl₂, at 0-500 mg/kg. At harvest, growth parameters, chlorophyll, and essential elements were determined. None of the treatments affected germination and pod production, and only ZnCl₂ at 250 and 500 mg/kg reduced relative chlorophyll content by 66 and 54%, respectively. While Z-COTE[®] did not produce phenotypic changes, Z-COTE-HP1[®], at all concentrations, increased root length (~53%) and leaf length (~18%) at 125 and 250 mg/kg, compared with control. Bulk ZnO reduced root length (~53%) at 62.5 mg/kg and ZnCl₂ reduced leaf length (~24%) at 125 mg/kg. Z-COTE[®], at 125 mg/kg, increased Zn by 203%, 139%, and 136% in nodules, stems, and leaves, respectively; while at the same concentration, Z-COTE-HP1[®] increased Zn by 140%, 39%, and 105% in roots, stems, and leaves, respectively. At 125 mg/kg, Z-COTE-HP1[®] increased root S (65%) and Mg (44%), while Z-COTE[®] increased stem B (122%) and Mn (74%). Bulk ZnO and ZnCl₂ imposed more toxicity to kidney bean than the NMs, since they reduced root and leaf elongation, respectively, and the concentration of several essential elements in tissues.

Keywords: ZnO nanomaterials, Surface coating, Red kidney bean, Plant growth, Essential elements

1. Introduction

Zinc oxide (ZnO) is widely used in commercial products, especially in personal care and cosmetics (PC&C). ZnO is incorporated as nanomaterial (NM) in several applications due to its improved properties, compared to the bulk counterpart.

The global demand for ZnO NMs brought its production to 33,400 tons in 2012, representing 2.4% of overall commercial ZnO [1]. Among the commercially available ZnO NMs, Z-COTE[®] and Z-COTE HP1[®] are used in numerous PC&C, especially in sunscreens, due to the enhanced UV absorbing properties and negligible scattering of visible light, which make them nearly transparent [2,3]. Z-COTE[®] is an amphiphilic uncoated ZnO NM, while Z-COTE HP1[®] is hydrophobic, coated with triethoxycaprylylsilane.

Agricultural fields are exposed to NMs through irrigation with contaminated water from manufacturing plants or domestic greywater, fertilization with NM-containing sludge, environmental nano-remediation, and accidental release of NM from industrial sites [4]. In addition, intentional use of nano-agricultural products represents a potential pathway for plant exposure to NMs [4–6].

Assessment of ZnO NMs in terrestrial plants include foliar or root exposure through hydroponic or soil experiments. Reports from hydroponics studies have showed effects in germination [7,8], root elongation [7,9,10], genotoxicity and cytotoxicity [11], and Zn biotransformation [12,13]. Soil studies revealed contradictory effects: negative impacts on plant growth [14–16] and yield [17], positive effects on growth [18,19], chlorophyll content, and fruit yield [18], and changes in the nutritional composition of plant tissues [20].

Zinc accumulation in plants exposed to ZnO NMs had shown concentration-dependent trends [10,13,16–22]. However, a considerable number of studies have evaluated in-lab synthesized instead of commercially produced NMs. To the best of the authors' knowledge, none of the

reported studies have assessed the effects of commercial particles such as Z-COTE[®] or Z-COTE HP1[®] in any plant species.

This study was aimed at comparing the effects of Z-COTE[®] and Z-COTE HP1[®] in the physiology and nutrient composition of soil grown bean plants. Bulk ZnO and ZnCl₂ were used for comparison purposes. Seed germination, biomass production, relative chlorophyll content, and pod production were recorded after 45 days of exposure, in addition to quantification of Zn, micro, and macroelements in plant systems.

2. Materials and methods

2.1 Zinc oxide nanomaterials/compounds and bean seeds

Z-COTE[®] and Z-COTE HP1[®] were purchased from BASF. These NMs were selected due to their extensive commercial applications, and because previous researchers [2], when characterizing NMs for the Organisation for Economic Cooperation and Development (OECD), included Z-COTE[®] and Z-COTE HP1[®] as two ZnO NMs representative of commercially available products. Bulk ZnO ACS reagent $\geq 99.0\%$ purity was purchased from Sigma-Aldrich and ZnCl₂ ACS reagent $\geq 97+\%$ purity from Acros Organics. Common bean (*Phaseolus vulgaris* L. var. red hawk kidney) seeds were provided by Dr. James Kelly, Michigan State University, stored at 4°C and rinsed before use with 2% NaClO for disinfecting purposes.

2.2 Physicochemical characterization of ZnO NMs

Z-COTE[®] and Z-COTE HP1[®] were characterized following the methods described in the supplementary information.

2.3 Soil amendment and seeds exposure

Natural soil was collected from a local farm in Socorro, TX, 79927, USA and characterized according to the Wentworth size classification (Medium loam: 19% clay, 44% silt, and 36% sand; 2.8% organic matter, pH = 7.825 ± 0.021 , EC = $1705 \pm 47.6 \mu\text{S/cm}$, and TDS = $847.5 \pm 23.8 \text{ mg/L}$).

Four replicates of the Z-COTE[®], Z-COTE HP1[®], bulk ZnO, and ZnCl₂ suspensions/solutions at 62.5, 125, 250 and 500 mg/kg soil (compound based) were prepared. Before use, soil was passed through an 8 mm sieve. Powders of both Z-COTE[®] and bulk ZnO were suspended in 100 mL of deionized water (DIW) and bath sonicated (Crest Ultrasonics, Trenton, NJ) for 30 min at 25 °C and intensity of 180 watts. ZnCl₂ and Z-COTE HP1[®] were mixed with no sonication. The suspensions/solutions were added to 1.3 kg of soil, mixed with a hand cultivator until homogeneity and transferred into plastic pots (12.5 cm diameter $\times$ 14 cm height). Acid washed commercial gravel (130 g of 1.0-1.5 mm diameter, 5% HNO₃) was placed at the bottom of each pot to enable aeration. Four replicates of soil mixed with deionized water were used as control for each compound. The experiment included 80 pots, 16 for each Zn compound and 16 for controls.

After 24 hours, five bean seeds were planted equidistantly in each pot at 2.5 cm depth, watered with 50 mL of DIW and transferred to a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) with 14 h photoperiod ($340 \mu \text{ mole m}^{-2} \text{ s}^{-1}$), 25/20 °C day/night temperature and 65-70% relative humidity.

2.4 Plant growth and chlorophyll production

Pots were watered daily with 50 mL until germination; then with 100 mL for 45 days, when 50% of control pods were fully developed. At harvest, relative chlorophyll content was measured in the center of the lowest trifoliate leaf (five plants/replicate) using a single-photon avalanche diode (SPAD) chlorophyll meter (Minolta, Japan SPAD, Spectrum Technologies). Number of plants, mature, and immature pods were recorded. Each plant was removed from the soil and severed at the crown. Trifoliate leaves (including the petiole) and pods were separated from the stems. Unifoliate leaves and flowers were discarded. Tissues were weighed and washed three times with 0.01M HNO₃ and deionized water (18 MΩ) alternately, and lengths were measured. For leaf length, measurement was taken along the central vein of the three longest leaves of each plant. Roots were frozen at -2 °C in plastic bags until processed. Nodules were separated from the roots and weighed. Tissues were oven dried in paper envelopes for 72 h at 70 °C and the dry weight was recorded.

2.5 Elemental quantification via ICP-OES

Ground dry samples were acid digested and analyzed for macronutrients (Ca, Mg, P, S) and micronutrients (Zn, Fe, B, Mn, Mo, Cu, Ni) following the methods reported by Peralta-Videa [20].

2.6 Statistical analysis

Data was analyzed using the Statistical Package for the Social Sciences 22 (SPSS, Chicago, IL, USA). One-way ANOVA was utilized to evaluate the experimental variance and differences between treatments were scrutinized with the multi comparison Tukey's HSD test at a *p*-value of 0.05. Data presented are means ± standard errors (SE) of specified number of replicates.

3. Results and discussion

3.1 Physicochemical characterization of ZnO NMs

Comprehensive physicochemical characterization data of ZnO NMs is shown in Table S1 and Figure S1 (Supplementary material).

3.2 Seed germination and pod production

Table S2 shows the data for seed germination and pod production. Pods were classified as immature if they appeared empty, while mature pods had seeds. As seen in Table S2, none of the nanomaterials/compounds show significant differences in seed germination and pod production, compared to controls. Furthermore, seed production was not compromised.

3.3 Effects on plant growth

Figure 1 displays phenotypical changes in bean plants exposed to the different Zn compounds. As seen in Figure 1a, at 62.5 mg/kg, bulk ZnO reduced root length (53%), while Z-COTE HP1[®], at all concentrations, increased it (up to 53%), compared with control. Previous studies reported reduction in alfalfa root length exposed to bulk ZnO at 500 mg/kg [15], as well as in tomato root length exposed to ZnO NM at 250 mg/kg and above [18] and buckwheat roots at 100 mg/kg [14]. However, other studies have shown contrary results. ZnO NM at 500 mg/kg, increased soybean root length [12], and at 0-500 mg/kg, green pea root length [19]. In the current study, Z-COTE HP1[®], at all concentrations, increased bean root size ($p \leq 0.05$). The Z-COTE HP1[®] coating has Si in the surface, which is known to promote root growth, even in plants for which Si is not essential [23].

None of the NMs affected stem elongation (Figure 1b); however, bulk ZnO, at 250 and 500 mg/kg, increased stem length by 30% and 31%, while ZnCl₂ at 500 mg/kg reduced it by 25%, compared with control. Similar results have been reported in alfalfa [15] and in green pea [19]. Mukherjee et al. [19] associated the shoot length promotion in green pea with the low oxidative radicals produced by bulk ZnO, and the toxicity of the ZnCl₂, with the high Zn accumulation in tissues, which was corroborated in the present study (Figure 3).

Figure 1c shows the data for leaf elongation. As seen in this figure, Z-COTE[®] did not affect leaf length; however, compared with control, Z-COTE HP1[®] at 125 mg/kg and bulk ZnO at 125 mg/kg and above, increased leaf length (up to 24%) ($p \leq 0.05$). Oppositely, ZnCl₂ shortened leaf length by 24%, 20%, and 32% at 62.5, 250, and 500 mg/kg, respectively. The effects of Z-COTE HP1[®] could be produced by Si in the surface coating [23], and the effect of bulk ZnO could be attributed to a lower production in reactive oxygen species [19]. Negative effects of ZnCl₂ might be attributed to an excess of chloride in leaves, which has shown to decrease leaf size in beans [24].

3.4 Biomass production

The biomass data is shown in Table S2. As seen in this table, none of the ZnO NMs had an effect on the fresh and dry weights. However, bulk ZnO at 250 mg/kg decreased root dry weight by 39%, compared with control. Perhaps at this concentration, bulk ZnO aggregates reduced cell elongation at the germination stage, interfering with the formation of tonoplast intrinsic protein 3 (TIP3) aquaporins [25]. Additionally, ZnCl₂ at 500 mg/kg decreased fresh and dry weight in leaves by 65 and 57%, respectively, and by 39% in stems dry weight. These changes may be attributed to the toxicity of Cl⁻, which has shown to decrease plant growth and yield [24,26,27]. Chloride has shown toxicity to photosystem II, which affects the whole plant system [26]. ZnCl₂

significantly reduced alfalfa biomass [15]. In addition, Wang et al. [28] reported that ZnCl_2 was more toxic to cowpea than ZnO NMs.

Figure 1. Length of (a)root, (b)stem, and (c)leaf tissues of bean plants exposed to Z-COTE[®], Z-COTE HP1[®], bulk ZnO, and ZnCl_2 at 0 (control), 62.5, 125, 250 and 500 mg/kg of soil. Values are means $\pm$ SE of 4 replicates for Zn compounds and 16 for controls. Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$).

3.5 Effects of treatments on relative chlorophyll content in leaves

Figure 2 shows the relative chlorophyll content of kidney bean plants exposed to the Zn NMs or compounds. Opposite to Mukherjee et al. [19] that reported reduced relative chlorophyll in peas exposed to ZnO NMs and bulk ZnO, none of the ZnO treatments affected the relative chlorophyll content in kidney bean plants. On the other hand, ZnCl₂ at 250 and 500 mg/kg decreased relative chlorophyll content by 66 and 54%, with respect to control ($p \leq 0.05$). Chloroplasts are highly permeable to Cl⁻ [29], which reduces the photosynthetic capacity as a result of chlorophyll degradation caused by an impact in photosystem II [24,26].

Figure 2. Relative chlorophyll content of bean leaves exposed to Z-COTE[®], Z-COTE HP1[®], bulk ZnO, and ZnCl₂ at 0 (control), 62.5, 125, 250 and 500 mg/kg of soil. Values are means $\pm$ SE of 4 replicates for Zn compounds and 16 for controls. Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$).

3.6 Zinc uptake and translocation

Similarly to previous studies [10,13,16–22], kidney bean plants accumulated Zn in a concentration-dependent manner, after exposure to Z-COTE[®] and Z-COTE HP1[®] at

concentrations of 125 mg/kg and above (Figure 3). As expected, there was higher Zn accumulation in the root system.

All roots exposed to Z-COTE[®] had more Zn in nodules ($p \leq 0.05$), compared with control and the other treatments (Figure 3a). Increases in nodule Zn under Z-COTE[®] varied from 100% at 62.5 mg/kg to 850% at 500 mg/kg, followed by Z-COTE HP1[®] at 250 and 500 mg/kg (166% and 461%, respectively), and ZnCl₂ at 500 mg/kg (285%), compared with control plants (Figure 3a). None of the bulk ZnO concentrations increased Zn in nodules, compared with control. This could be an effect of particle size (<1000 nm, [30]) and aggregation.

In the root system, none of the treatments at the 62.5 mg/kg affected Zn accumulation (Figure 3b); very likely due to fixation of Zn in the soil matrix [31]. At 125 mg/kg and above, all compounds, except bulk ZnO, produced higher root Zn ($p \leq 0.05$), compared with control. It is possible that the high particle size (<1000 nm) of bulk ZnO avoided the uptake [30]. Zn accumulation in roots from ZnO NMs and ZnCl₂ showed different tendency at 125 mg/kg than at 250 and 500. However, the statistical comparisons of treatment means were clearly defined only at the highest concentration. At 500 mg/kg, root Zn was similar for ZnO NMs and ZnCl₂ (Figure 3b). It is premature to get a conclusion from this single work, but there is the possibility that this was due to the slow release of Zn from NMs over a long time exposure [32,33]. In addition, Wu et al. [34] mentioned that ZnO NMs attach to the cell surface, enhancing the transport of ionic Zn.

Zinc concentrations in stems followed a similar trend than in roots (Figure 3c). However, at the highest treatment concentration (500 mg/kg) the stem Zn in plants exposed to ZnCl₂ was significantly higher ($p \leq 0.05$), compared with the other compounds. This was expected, since ionic Zn is taken up as Zn(II), which is easily translocated by the ZIP transporters [35].

The Zn accumulation in leaves was similar to that in stems, except at 62.5 mg/kg, where accumulation from Z-COTE[®], Z-COTE HP1[®] and ZnCl₂ was significantly higher (89, 96, and 74%, respectively), compared with control ($p \leq 0.05$) (Figure 3d). Higher leaf Zn was found at 500 mg/kg of bulk ZnO (115%) and Z-COTE HP1[®] (205%), compared with control. More studies are needed to explain these results. However, it is hypothesized that smaller unaggregated coated particles are absorbed by plants and a portion of them is translocated to the leaves, while ionic Zn is complexed and retained in underground tissues. This hypothesis could be supported by higher Zn translocation factors reported for alfalfa leaves exposed to NMs, compared with ZnCl₂ [15].

Of particular interest is the Zn accumulation in pods (Figure 3e). At 62.5 mg/kg, none of the NMs or compounds reduced Zn accumulation in pods. Conversely, plant exposure to bulk ZnO at 125 mg/kg and above, resulted in a significant increase in pod Zn, compared with control ($p \leq 0.05$). Increases ranged from 67% at 125 mg/kg to 71% at 500 mg/kg. On the other hand, under exposure to the nanoparticulate forms, pod Zn increased, significantly, only at 500 mg/kg of uncoated Z-COTE[®]. Results suggest that none of the compounds downregulated the genes involved in Zn transport associated genes in developing pods [36].

3.7 Effects in the nutrient composition of roots, stems, leaves and pods

3.7.1 Effects in macronutrient composition

Accumulation of macroelements altered by Zn treatments is shown in Table 1. At 62.5 mg/kg, none of the nanomaterials affected macroelements' uptake and translocation, respect to control. In addition, none of the Z-COTE[®] concentrations perturbed the accumulation of nutritional elements in vegetative tissues. However, different effects were recorded for the coated NM, bulk ZnO, and ZnCl₂.

Coated Z-COTE HP1® at 125 and 500 mg/kg, increased Mg in roots by 65.1% and 69.1%, respectively. Magnesium is a mobile cation in soils, which was possibly attracted by the negative surface of Z-COTE HP1® (-23.6 ± 0.9 mV), favoring a simultaneous uptake [37]. Sulfur, which is available to plants in the form of anionic sulfate (SO_4^{2-}) [38], was augmented 64.7% in roots exposed to 125 mg/kg of Z-COTE HP1®, compared with control. Several transporters in the plasma membrane participate in the uptake and translocation of SO_4^{2-} , in addition to the activity of a proton pump maintained by ATPase. It is possible that the negatively charged surface of Z-COTE HP1® affected the proton ATPase activity and the transport channels [38], increasing the S uptake. The fact that only the 125 mg/kg treatment showed an increase in S could be related to the specificity of the high-affinity transporters that operate at low concentration of sulfate in the roots [39].

Bulk ZnO significantly reduced Ca in stems and leaves, compared with control. In stems, Ca was reduced 42.3% at 500 mg/kg; in leaves, 29.7% at 250 and 28.5% at 500 mg/kg. Magnesium was also diminished in leaves of plants exposed to bulk ZnO at 62.5 and 250 mg/kg (29.2 and 35.6%, respectively). It has been reported that the long distance transport of Mg is mediated by the *AtCNGC10* transporter that also participates in Ca transport [40]; thus, it is possible that large bulk particles could interfere with expression of this transporter; or a physical obstruction at phloem level could have occurred [41].

ZnCl_2 , at the highest concentration increased Ca in roots and stems by 32.0% and 52.7% respectively, compared with control. According to Hajrasuliha [24], Cl^- leads to higher accumulation of Ca, which explains the increased levels of this element in roots and stems. At such concentration, ZnCl_2 also increased Mg in leaves (36.1%). It has been shown that the long distance transport of Ca^{2+} is also facilitated by the same Mg^{2+} transporter [42]; thus ZnCl_2 affected the accumulation of both ions. In addition, Cl^- acts as a Mg^{2+} accompanying anion; thus,

Figure 3. Zinc uptake of (a)nodules, (b)roots, (c)stems, (d)leaves, and (e)pods of bean plants exposed to Z-COTE[®], Z-COTE HP1[®], bulk ZnO, and ZnCl₂ at 0 (control), 62.5, 125, 250 and 500 mg/kg of soil. Values are means $\pm$ SE of 4 replicates for Zn compounds and 16 for controls. Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$).

they might be taken up together [37]. Finally, ZnCl_2 at 500 mg/kg, increased P in stems (37.7%) and leaves (63.0%), compared with control plants. According to Zhu et al. (2001), Zn supply may affect the expression of P uptake efficiency. ZnCl_2 treated plants had 427% and 372% more Zn in stems and leaves than control plants, respectively, which could induce the increase in P uptake. Finally, none of the compounds disturbed the macronutrient concentration in pods.

3.7.2 Effects in micronutrient accumulation

Table 2 shows concentrations of micronutrients affected by Zn treatments. Overall, the impact was higher in the translocation than in the absorption. At absorption level, ZnCl_2 at 500 mg/kg reduced Mo uptake by 95%, Z-COTE HP1[®] increased B by 114% (250 mg/kg) and Mo by 76% (500 mg/kg); and Z-COTE[®] at 250 mg/kg increased B by 121%, compared with control.

Molybdenum is taken by plants as MoO_4^{2-} [44]; however, it forms ZnMoO_4 with Zn ions, which is insoluble in water [45]; thus, its absorption was reduced in plants exposed to ZnCl_2 . On the other hand, the negative surface of Z-COTE HP1[®] could repel the negative molybdate ions; with the possibility of triethoxycaprylsilane upregulating the MOT1 transporter in the root cell plasma membrane, increasing Mo uptake [46]. Boron is taken up as boric acid B(OH)_3 by diffusion and specific transporters. Several soil factors can interact with B uptake mechanism, such the negative surface of Z-COTE HP1[®] (-23.6 ± 0.9 mV) that may attract B, enhancing its uptake [47]. However, since both ZnO coated and uncoated NMs increased B uptake at similar rate, it is possible that these NMs upregulate B transporters in a similar way [48].

The translocation of microelements to stems and leaves was not affected by Z-COTE HP1[®] but the other compounds had different impacts on microelements compartmentalization in aboveground tissues. Results suggest that the translocation of cations or anions to the aerial part was not influenced by the negative surface charge of the coated NM. Uncoated Z-COTE[®] impacted the accumulation of B and Mn in stems. Boron increased by 122% and 116% at 125

and 250 mg/kg, respectively, while Mn was 74% higher (at 125 mg/kg) than control. B is absorbed from the soil following the water flow through the roots [49], and then it is transported to the xylem by a facilitating boron transporter 1 (BOR1) [50] for further distribution. Consequently, similarly to what occurred in root absorption, the uncoated NM probably upregulated the B transporters. Regarding Mn, several transporters have been implicated in its transport, including members of the ZIP (zinc-regulated transporter/iron-regulated transporter (ZRT/IRT1)-related protein) transporter family [51]. An increase in the available Zn from Z-COTE[®] can rise the activity of such transporters, enhancing Zn and Mn uptake.

Bulk ZnO impacted the most the micronutrient composition of stems and leaves. Mn and Cu were similarly reduced in both tissues, with reductions of up to 52% for Mn in leaves and 63% for Cu in stems. Manganese was reduced at all concentrations in leaves; while in stems, only at 250 and 500 mg/kg, it was statistically reduced, compared with control. It is possible that the adsorption of Mn by the negatively charged wall constituents of the root cell apoplastic spaces [52] did not occur due to the presence of bulk ZnO agglomerates.

Decreased levels of Cu were found in stems at all bulk ZnO concentrations, with values between 46% and 63% smaller than control. In leaves, Cu was reduced by 32% and 40% at 250 and 500 mg/kg, respectively, compared with control. It has been reported that high levels of Zn can reduce Cu uptake, by an antagonist interaction [27], due to both elements sharing the same transporters [53].

At 250 and 500 mg/kg, bulk ZnO reduced B in stems by 81% and 91%, compared with control. In this study, there was no Zn-deficiency that has been reported to increase B [54]; thus, it is hypothesized that the reduction in stem B was due to an antagonistic effect between B and Zn at translocation level.

Table 1. Macronutrients of roots, stems and leaves of bean plants altered after exposure to Z-COTE[®], Z-COTE HP1[®], bulk ZnO, and ZnCl₂ at 0 (control), 62.5, 125, 250 and 500 mg/kg of soil. Values are means $\pm$ SE of 4 replicates for Zn compounds and 16 for controls. Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$). (+) or (-) signs represent increase or decrease in nutrient concentrations, compared to the respective controls ($p \leq 0.05$).

Tissue	Nutrient	Treatment	Concentration (mg/kg dry tissue)				
			62.5 mg/kg	125 mg/kg	250 mg/kg	500 mg/kg	
Leaf	Ca	Control	29606 ± 1044 ab	29606 ± 1044 ab	29606 ± 1044 b	29606 ± 1044 bc	
		Bulk ZnO	23525 ± 1141 a	30382 ± 1548 b	20811 ± 3694 a-	21182 ± 3866 a-	
		Z-COTE®	25207 ± 282 ab	26617 ± 817 ab	28299 ± 1043 ab	27165 ± 1749 abc	
		Z-COTE HP1®	25422 ± 580 a	23589 ± 1408 a	23876 ± 723 ab	22467 ± 682 ab	
		ZnCl₂	31420 ± 1417 ab	30194 ± 674 ab	30985 ± 2382 b	32106 ± 1537 c	
	Mg	Control	6578 ± 277 bc	6578 ± 277 ab	6578 ± 277 bc	6578 ± 277 a	
		Bulk ZnO	4657 ± 276 a-	6681 ± 327 b	4234 ± 823 a-	4669 ± 1003 a	
		Z-COTE®	6010 ± 163 ab	6404 ± 416 ab	6431 ± 173 bc	6348 ± 239 a	
		Z-COTE HP1®	5452 ± 151 ab	4819 ± 381 a	5696 ± 197 ab	4885 ± 356 a	
		ZnCl₂	7746 ± 310 c	7039 ± 330 b	8297 ± 122 c	8950 ± 467 b+	
	P	Control	1027 ± 79 ab	1027 ± 79 ab	1027 ± 79 ab	1027 ± 79 a	
		Bulk ZnO	811 ± 85 a	1278 ± 100 ab	703 ± 59 a	727 ± 34 a	
		Z-COTE®	867 ± 49 a	818 ± 72 a	832 ± 38 a	956 ± 39 a	
		Z-COTE HP1®	879 ± 59 a	771 ± 81 a	745 ± 34 a	678 ± 15 a	
		ZnCl₂	1638 ± 432 b	1386 ± 147 b	1646 ± 369 b	1674 ± 118 b+	
	Stem	Ca	Control	11265 ± 540 a	11265 ± 540 ab	11265 ± 540 ab	11265 ± 540 b
			Bulk ZnO	8755 ± 839 a	8475 ± 570 a	8792 ± 470 a	6504 ± 420 a-
			Z-COTE®	11219 ± 101 a	11999 ± 334 b	11345 ± 896 ab	10343 ± 790 b
			Z-COTE HP1®	10267 ± 500 a	10068 ± 584 ab	10014 ± 623 ab	10272 ± 551 b
			ZnCl₂	9559 ± 2042 a	12950 ± 259 b	13501 ± 690 b	17202 ± 476 c+
P		Control	748 ± 46 a	748 ± 46 ab	748 ± 46 ab	748 ± 46 a	
		Bulk ZnO	611 ± 66 a	468 ± 23 a	666 ± 8 a	509 ± 40 a	
		Z-COTE®	708 ± 36 a	789 ± 92 ab	613 ± 44 a	752 ± 42 ab	
		Z-COTE HP1®	738 ± 37 a	689 ± 57 ab	744 ± 31 ab	664 ± 23 a	
		ZnCl₂	964 ± 321 a	963 ± 107 b	970 ± 67 b	1030 ± 48 b+	
Root	Ca	Control	17153 ± 815 a	17153 ± 815 a	17153 ± 815 a	17153 ± 815 a	
		Bulk ZnO	18463 ± 1366 a	17233 ± 2541 a	16505 ± 680 a	19381 ± 1276 ab	
		Z-COTE®	19442 ± 1029 a	22011 ± 759 a	20075 ± 1662 a	19383 ± 548 ab	
		Z-COTE HP1®	17866 ± 1004 a	17398 ± 853 a	17172 ± 1027 a	16717 ± 765 a	
		ZnCl₂	19858 ± 594 a	18897 ± 849 a	17465 ± 746 a	22642 ± 504 b+	
	Mg	Control	5481 ± 553 a	5481 ± 553 ab	5481 ± 553 a	5481 ± 553 a	
		Bulk ZnO	5106 ± 1012 a	4396 ± 523 a	4697 ± 294 a	5340 ± 982 a	
		Z-COTE®	5972 ± 386 a	7081 ± 438 abc	7826 ± 248 a	6546 ± 422 ab	
		Z-COTE HP1®	6695 ± 1622 a	9061 ± 552 c+	7333 ± 1024 a	9268 ± 277 b+	
		ZnCl₂	6057 ± 779 a	7926 ± 562 bc	6471 ± 281 a	5349 ± 231 a	
	S	Control	4295 ± 461 a	4295 ± 461 a	4295 ± 461 a	4295 ± 461 ab	
		Bulk ZnO	3960 ± 947 a	4147 ± 345 a	3805 ± 247 a	4582 ± 905 ab	
		Z-COTE®	4206 ± 199 a	5079 ± 444 ab	6272 ± 574 a	4281 ± 425 ab	
		Z-COTE HP1®	5148 ± 1307 a	7076 ± 353 b+	5732 ± 1265 a	6260 ± 522 b	
		ZnCl₂	4896 ± 719 a	6854 ± 213 ab	5014 ± 426 a	2049 ± 194 a	

Bulk ZnO was the only compound affecting Ni. Plants exposed to 62.5 and 500 mg/kg had, respectively, 60 and 87% less Ni in stems than control plants. Cataldo et al. [55] suggested that in soybean seedlings, Ni^{2+} and Zn^{2+} are absorbed using the same carrier site, since the presence of Zn^{2+} inhibited the uptake of Ni^{2+} . Although something similar could occur in kidney bean, it is interesting that only bulk ZnO caused this effect, suggesting an effect of the particle size.

ZnCl_2 increased the translocation of Mn, Fe, Cu, Mo, Mn, and Ni. Very likely, Cl^- is responsible for increased transportation of these ions

Increased Fe concentrations were found in stems and leaves (92 and 61% higher than control) and Mn in leaves (31%) of plants exposed to 500 mg/kg of ZnCl_2 . Since ZnCl_2 is water soluble, both ions will be available to bind other ions/molecules and be transported, suggesting that Fe could bind Cl^- at root level, forming colloidal particles. A previous report had indicated that plants are able to take Fe from colloids [56]. In case of Mn, Westermann et al. [57] indicates that the addition of Cl to the soil increases Mn uptake by roots.

Conversely to what happened in roots, ZnCl_2 at 500 mg/kg increased Mo in stems by 92%. The mechanisms of Mo transport in plants are still elusive [58]. However, there is strong evidence that Mo and S compete for the same transporter [58]. As seen in Table 1, ZnCl_2 at 500 mg/kg resulted in an important reduction of root S (52%). This suggests that the S transporter was more available for Mo transport, culminating in an increase of Mo in stems (Table 2).

Finally, pods did not show statistical difference in microelement concentrations after exposure to Zn NMs and compounds, compared to control.

3.7.3 Effects in nodules' nutrients

Figure S2 shows the statistically significant changes in nodules' nutrients of Zn treated plants. Among the tested compounds, only bulk ZnO showed significant effects. Bulk ZnO decreased

Ca (as low as 79%) and Ni (as low as 60-100%), at all concentrations, compared with controls. Changes in Ca concentration inside the cells trigger a signaling cascade that involves expression of genes responsible for nodule development and symbiotic activity [59]. Nickel has been regarded as an important element in the symbiotic relationship between soybean plant and its rhizobia (*Rhizobium japonicum*) [60]. Therefore, the impact of the ZnO could represent a potential risk for the nodule's activity, caused by changes in accumulation of nutrients such as Ca and Ni.

Manganese was also reduced by bulk ZnO at 125 mg/kg by 99.8%, as well as P at 125 mg/kg and above, with values of 65, 63 and 52% lower than controls. P showed to play specific roles in nodule initiation, growth, and functioning in soybean [61], and in *Medicago truncatula*, insufficient P reduced early nodule functioning, along with increased release of protons in roots [62]. According to O'Hara [63], deficiencies in Ca, Fe, and Mo could cause dysfunctional nodule development and activity.

4. Conclusions

This study aimed to determine the effects of uncoated and coated ZnO NMs in kidney bean plants. Effects were compared to the corresponding bulk counterpart, ionic zinc and control plants. Results showed that Z-COTE HP1[®] promoted root growth, while no effects were found for Z-COTE[®]. On the other hand, ZnCl₂ at the highest concentration significantly diminished leaf length, fresh and dry weight of leaves, and relative chlorophyll content, suggesting that this compound was more toxic than the NMs.

Zinc accumulation was differentially affected by the compounds. Uncoated ZnO, at all concentrations, and coated ZnO at 250 and 500 mg/kg, increased nodule Zn. While bulk ZnO reduced nodule Zn at all concentrations. In leaves, uncoated ZnO at 500 mg/kg resulted in

significantly more Zn, compared with coated ZnO. At 500 mg/kg, bulk ZnO, Z-COTE[®], and ZnCl₂ increased pod Zn by 60%, 50, and 35%, respectively.

The ZnO NMs also affected the homeostasis of plant ionome. Compared with control, Z-COTE HP1[®] increased B, Mo, Mg, and S in roots, while Z-COTE[®] increased B in roots stems, and Mn in stems. Bulk ZnO reduced Cu and Mn in stems and leaves. Nodules' composition was only affected by bulk ZnO (decreased Ca, Ni, Mn, and P). ZnCl₂ affected nutrients in roots, stems and leaves, mainly increasing them. Nutrients in pods, except Zn, were not affected by any treatment.

Overall, coated ZnO increased root length and the concentration of more nutritional elements than uncoated Z-COTE[®]; however, none of them affected pod production. Bulk ZnO and ZnCl₂ imposed more toxicity to kidney beans, since they reduced root and leaf elongation, respectively, and several essential elements in tissues.

Table 2. Micronutrients of roots, stems and leaves of red kidney bean plants altered after exposure to Z-COTE[®], Z-COTE HP1[®], bulk ZnO, and ZnCl₂ at 0 (control), 62.5, 125, 250 and 500 mg/kg of soil. Values are means $\pm$ SE of four replicates for Zn compounds and 16 for controls. (+) or (-) signs represent increase or decrease in nutrient concentrations, compared to the respective controls ($p \leq 0.05$).

Tissue	Nutrient	Treatment		Nutrient concentration		% change
		Compound	mg/kg	mg/kg dry tissue	$\pm$ std. error	
Leaf	Mn	Control		98.6 $\pm$ 4.9		100 %
		Bulk ZnO	62.5	57.8 $\pm$ 3.8		41 % -
			125	54.9 $\pm$ 3.1		44 % -
			250	47.1 $\pm$ 14.7		52 % -
			500	53.3 $\pm$ 14.4		46 % -
		ZnCl ₂	500	128.9 $\pm$ 13.5		31 % +
	Fe	Control		58.3 $\pm$ 2.7		100 %
		ZnCl ₂	500	94.0 $\pm$ 18.3		61 % +
	Cu	Control		6.5 $\pm$ 0.3		100 %
		Bulk ZnO	250	4.4 $\pm$ 0.2		32 % -
			500	3.9 $\pm$ 0.3		40 % -
		ZnCl ₂	500	8.7 $\pm$ 0.6		34 % +
Stem	Fe	Control		54.6 $\pm$ 3.6		100 %
		ZnCl ₂	500	104.8 $\pm$ 21.3		92 % +
	Mo	Control		51.2 $\pm$ 3.8		100 %
		Bulk ZnO	500	77.8 $\pm$ 10.1		52 % +
		ZnCl ₂	500	98.3 $\pm$ 9.0		92 % +
	B	Control		21.6 $\pm$ 2.2		100 %
		ZnO Bulk	250	4.1 $\pm$ 0.1		81 % -
			500	1.9 $\pm$ 0.3		91 % -
		Z-COTE [®]	125	48 $\pm$ 15.3		122 % +
			250	46.6 $\pm$ 2.5		116 % +
	Mn	Control		14.6 $\pm$ 1.3		100 %
		Bulk ZnO	250	5.5 $\pm$ 0.7		62 % -
			500	7.7 $\pm$ 0.3		47 % -
		Z-COTE [®]	125	25.4 $\pm$ 1.1		74 % +
	Cu	Control		5.2 $\pm$ 0.3		100 %
		Bulk ZnO	62.5	2.8 $\pm$ 0.4		46 % -
			125	1.9 $\pm$ 0.2		63 % -
			250	2.8 $\pm$ 0.3		46 % -
			500	1.9 $\pm$ 0.1		63 % -
		ZnCl ₂	500	6.8 $\pm$ 0.3		31 % +
	Ni	Control		1.5 $\pm$ 0.2		100 %
		Bulk ZnO	62.5	0.6 $\pm$ 0.2		60 % -
			500	0.2 $\pm$ 0.2		87 % -
		ZnCl ₂	500	2.2 $\pm$ 0.1		47 % +
Root	Mo	Control		16.0 $\pm$ 1.6		100 %
		Z-COTE HP1 [®]	500	28.1 $\pm$ 2.0		76 % +
		ZnCl ₂	500	0.8 $\pm$ 0.5		95 % -
	B	Control		8.5 $\pm$ 1.3		100 %
		Z-COTE [®]	250	18.8 $\pm$ 0.6		121 % +
		Z-COTE HP1 [®]	250	18.2 $\pm$ 1.9		114 % +

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