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# Exposure Of Kidney Bean (Phaseolus Vulgaris) Plants To Coated And Uncoated Zinc Oxide Nanomaterials Under Different Soil Conditions: Effects On Plant Growth And Seed Quality

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*I dedicate this work*

*TO GOD*

*for His innumerable blessings,*

*and especially*

*TO MY MOM*

*with love*

# EXPOSURE OF KIDNEY BEAN *(PHASEOLUS VULGARIS)* PLANTS TO COATED AND UNCOATED ZINC OXIDE NANOMATERIALS UNDER DIFFERENT SOIL CONDITIONS: EFFECTS ON PLANT GROWTH AND SEED QUALITY

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# Abstract

Soil exposure to engineered nanomaterials (NMs) occurs through the intentional use of nano-agrochemicals, incidental contamination from industrial-waste release, irrigation with wastewater and gray water, or amendment with NM-loaded sludge. Although there are several reports about the effects of NMs in terrestrial plants, there is still a lack of knowledge about the effects of NMs in crop plants and their edible portions.

Among the large list of produced NMs, it is estimated that 34,000 tons of ZnO NMs are produced yearly. Due to their growing applications in cosmetics and personal care products, there is a high possibility that the ZnO NMs released into the environment could reach edible crops. Even when research in the implications of ZnO NMs in crop plants is growing, to the best of the author's knowledge, none of the studies have focused on commercial Z-COTE and Z-COTE HP1 ZnO NMs. Z-COTE is an uncoated ZnO NM of hydrophilic nature, while Z-COTE HP1 is hydrophobic due to a surface coating of triethoxycaprylylsilane, both manufactured by BASF.

Amid the crops for human feed, beans are the most consumed legume in the world, with productions over 26 million tons in a large range of environments world-wide. Thus, there is a possibility that the large amounts of commercial ZnO NMs released into the environment could reach bean crops.

This research aimed at evaluating the effects of Z-COTE and Z-COTE HP1 on the development and yield of bean plants (*Phaseolus vulgaris* L. var. red hawk kidney). In this study, the long-term exposure of Z-COTE and Z-COTE HP1 to common bean *(Phaseolus vulgaris)* was evaluated through the effects on plant growth, yield, nutritional quality of seeds, and residual effects of these NMs in seeds from the second generation plants. The research was divided into three stages. Stage I was established to evaluate the effects of the ZnO NMs in the plant's physiological, biochemical, and agronomical parameters. Stage II was set to evaluate the effects of the ZnO NMs in mature seeds harvested from two different soils, while the residual effects of the ZnO NMs in seeds of second generation plants were

evaluated in stage III.

In stage I, bean plants were grown for 45 days in natural soil (NS) amended with either Z-COTE, Z-COTE-HP1, bulk ZnO, or ionic $ZnCl_2$ at 0, 62.5, 125, and 500 mg $kg^{-1}$. Growth parameters and essential elements were determined. Z-COTE did not produce phenotypic changes, while Z-COTE-HP1 increased root and leaf length. Z-COTE increased Zn in nodules, stems, and leaves, while Z-COTE-HP1 increased it in roots, stems, and leaves. At 125 mg/kg, Z-COTE-HP1 increased S and Mg in root, but Z-COTE increased stem B and Mn. Bulk ZnO and $ZnCl_2$ imposed more toxicity than the NMs, since they reduced root and leaf elongation, respectively, and the concentration of several essential elements in tissues.

In stage II, yield and seed nutrient composition were evaluated in plants grown to maturity in NS or organic matter-enriched soil (ES) amended with ZnO NMs, bulk ZnO or $ZnCl_2$. Results showed an interaction of soil $\times$ compounds that reduced the maturation time by 25 days and increased seed yield in ES, compared to NS. Z-COTE HP1 and $ZnCl_2$ produced the highest sugar content in seeds of plants grown in NS and ES, respectively. In addition, seeds from ES + Z-COTE HP1 had less Zn than the rest of the compounds. In comparison to NS, the organic matter enrichment and pH reduction in ES enhanced the accumulation of Zn, K, S, P, Mg, Ca, Fe, and Mn in seeds from all tested compounds, but reduced Mo under Z-COTE HP1 and $ZnCl_2$. In general, Z-COTE and Z-COTE HP1 affected seed nutritional elements in a similar manner. However, results indicate that the effects of ZnO NMs in bean plants vary with soil composition.

In stage III, seeds from plants cultivated in ES amended with 0-500 mg $kg^{-1}$ of the ZnO NMs were cultivated in ES without further exposure to ZnO NMs. At day 45, the activity of antioxidant enzymes in seeds was evaluated. At maturity, the yield and nutritional composition of seeds were assessed. None of the treatments affected the yield, sugar, protein, and the activities of catalase and ascorbate peroxidase. However, superoxide dismutase activity increased in seeds from the 500 mg $kg^{-1}$ $ZnCl_2$ treatment. There was no residual effect on Zn accumulation; however, Z-COTE and Z-COTE HP1 reduced Ni, compared

with control, suggesting epigenetic changes driven by the enhanced Zn accumulation in seeds from the previous generation.

This dissertation provided valuable insight into the interaction of Z-COTE and Z-COTE HP1 with bean plants. Overall results of this research have shown that, at the evaluated concentrations and growth conditions, Z-COTE and Z-COTE HP1 do not represent a threat for the growth and production of bean plants. These findings open an opportunity for application of ZnO NMs in agriculture.

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# Chapter 1

## Introduction

The enhanced properties that nanomaterials (NMs) offer provide a variety of applications in modern life products (Hong *et al.*, 2013). In 2001, the US created the National Nanotechnology Initiative, which increased the public funding for nanotechnology research (Roco & Alivisatos, 2000) at the National Science Foundation (NSF), where the nanoscale science and engineering research funding grew from $3M, in 1991, to more than $460M, in 2012 (Chen *et al.*, 2013). However, while the majority of research in nanotechnology focuses on the development of new technologies, only a small fraction of scientists focuses on the environmental implications of nanotechnology.

The increasing development of nanotechnologies has raised concerns regarding the NMs release and final fate into the environment and the consequences they may cause into living systems. In recent years, several researchers have been focusing on the evaluation of the effects of a large list of NMs in a variety of living organisms, including terrestrial and aquatic plants, bacteria, and small animals. As the inventory of NMs keeps growing, building a clear panorama about how these materials will affect the environment is essential.

## 1.1 Engineered nanomaterials

Nanomaterials can be defined as materials with at least one external dimension or an internal/surface structure ranging between 1 and 100 nm ASTM (2006). They can be classified based on their origin as referred by Mishra *et al.* (2014):

1. **Natural NMs**, which can be found naturally in the environment (e.g. volcanic dust and mineral composites).

2. **Incidental NMs**, produced as a result of some human activities (e.g. diesel exhaust and welding fumes).

3. **Engineered NMs**, manufactured to fill specific conditions. In this category, the carbon-based NMs (e.g. carbon nanotubes (CNTs) and fullerenes); metal-based NMs (e.g. ZnO and gold nanoparticles), dendrimers or nano-sized polymers; and composites, which are a combination of a nanoparticle and a larger material, are included.

The outstanding properties of engineered nanomaterials (NMs) start with a small size, which allows them to be easily internalized by biological structures along with a large surface area-to-volume ratio that makes them highly reactive. Some NMs are also recognized for their physical strength as well as electrical conductivity properties (Hong *et al.*, 2013). In some cases, the NMs show superior optical effects than their bulk counterparts. And finally, NMs can be functionalized, by coating or capping their surfaces in order to modify their chemical properties, such as dispersibility and conductivity (Peralta-Videa *et al.*, 2011).

NMs can be found in numerous products from diverse industries including agriculture, aerospace, automotive, catalysis, paints, construction, cosmetics, medicine, electronics, filtration, food, and textile (Keller *et al.*, 2013).

The release of NMs into the environment has urged the research community to build up the knowledge regarding the interaction and behaviour of these materials with their surroundings and the effects they can cause in their paths to their final destinations. Even when there is an increasing number of investigations on the implications of NMs in plants (Sardoiwala *et al.*, 2017, Tripathi *et al.*, 2017b, Zuverza-Mena *et al.*, 2017), aquatic organisms (Gupta *et al.*, 2017, Jahan *et al.*, 2017), and microorganims (Sardoiwala *et al.*, 2017, Shumayal *et al.*, 2018, Tripathi *et al.*, 2017a) there is not enough evidence of toxicity or harmlessness in order to establish regulations on the production, use, and disposal of NMs.

## 1.2 ZnO NMs: Z-COTE and Z-COTE HP1

Zinc oxide (ZnO) is a white chemical that can be found in rubber, glass products, paints, fertilizers, human and animal supplements, cosmetics, and medical products. The variety of applications arises from the different functions it can have, including: bulking agent, colorant, catalyst, essential trace element, corrosion inhibitor, mildew controller, antibacterial, and deodorant agent. One of the main uses for ZnO is in sunscreen lotions and other cosmetics and personal care products (PCPs), due to its strong absorption of UV light (it blocks UVA and UVB radiation); it is an excellent protector against sunburn and cancer (SCCS, 2012).

The broad-spectrum UV absorbing properties of ZnO are enhanced when synthesized as nanoparticles. The small size of ZnO NMs makes them transparent after skin application because of their "negligible scattering of visible light" (Yin *et al.*, 2015) as it can be noticed in Figure 1.1.

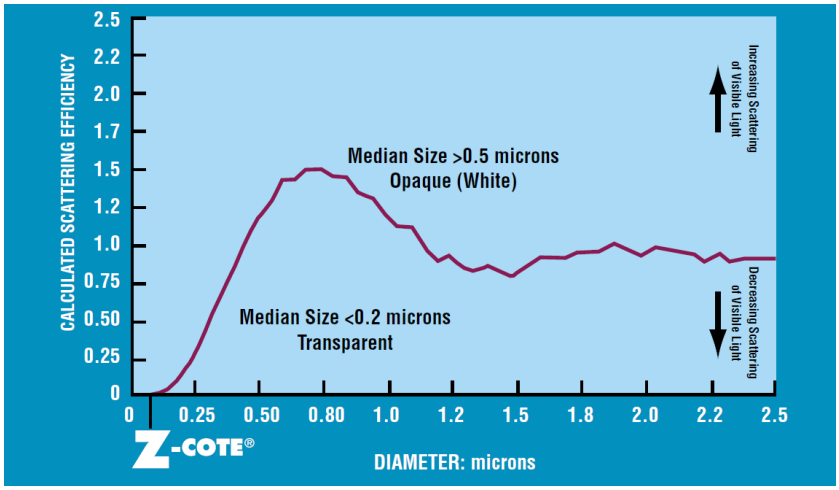


Figure 1.1: Scatter efficiency of Z-COTE nanomaterials, as advertised by the manufacturer. *Reproduced from BASF (2000).*

It is not only the application of ZnO NMs in PCPs which has built up the world production, but also newly developed technologies, based on the electronic and/or opto-

electronic properties, catalytic capacity, and UV-absorption of nanosized ZnO. Solar cells, laser diodes and a variety of coatings represent some examples of these applications (Yin *et al.*, 2015). It has been estimated that between 34,000 and 42,000 metric tons of ZnO NMs are produced yearly (Keller *et al.*, 2013), from which 1,800 to 2,100 metric tons/year are incorporated into PCPs.(Keller *et al.*, 2014).

Among the large list of commercially available ZnO NMs, Z-COTE and Z-COTE HP1 produced by BASF are used in numerous cosmetics from recognized brands, such as Ponds®, Olay® (SCCS, 2012), and Dermatone®. The exceptional properties attributed to ZnO NMs according to the manufacturer (besides those mentioned above) include stability against sun degradation, hypoallergenic, and non greasy (BASF, 2000). Additionally, the possibility of having two different ZnO NMs: Z-COTE, which is a bare ZnO NM of amphiphilic nature can be incorporated in water-based formulations; whereas, Z-COTE HP1 with a hydrophobic coating of triethoxycaprylylsilane is suitable for oil-based products.

There are scientific reports of some toxicity studies performed by the European Commission of Health and Food Safety (SCCS, 2012) concluding that the potential genotoxicity of ZnO NMs can not be yet stated. In addition, the same document enunciates that the risk assessment of nanomaterials presents some uncertainties in relation to the behaviour of the NMs in biological systems, and also highlights the limitations of the analytical methods used for toxicity tests that are designed for regular-sized materials. Finally, it is necessary to emphasize that these studies were performed in rats, rabbits, and similar animals as a way to understand the effects they might have on humans. To the best of the author's knowledge, there are no reported studies on plants exposed to Z-COTE and Z-COTE HP1.

According to Wang *et al.* (2013), plants can absorb NMs with a size smaller than 20 nm via plasmodesmata and endocytosis. Thus, plants represent a potential pathway to transport the different NMs through the food chain. However, to the best of the author's knowledge, there are no published studies of Z-COTE and Z-COTE HP1 in any terrestrial plant.

## 1.3 Terrestrial plants exposed to ZnO NMs

Due to the large production and applications of ZnO NMs, their release into the environment represents a potential pathway to reach agricultural soils and some edible plants. Exposure of NMs to soil may occur through the intentional use of nano-agrochemicals, incidental contamination from industrial-waste release, irrigation with wastewater and gray water, or amendment with NMs-loaded sludge (Medina-Velo *et al.*, 2017c).

Reports about the exposure of ZnO NMs to terrestrial plants include cultivation in solution (hydroponics), soil, and direct exposure. The following is a chronological summary of soil studies performed in natural soils, standard soils, commercial soils, and also in soil microcosm, the last ones aimed at evaluating the impact in the plant microbiota.

Hydroponic studies include an onion (*Allium cepa*) experiment performed by Ghodake *et al.* (2011), where plants exposed to ZnO NMs at concentrations of 5, 10, and 20 $\mu$g mL$^{-1}$ showed negative effects on root elongation, which diminished as the concentration of ZnO NMs increased. The authors pointed that ZnO NMs exerted phytotoxicity because they accumulated in the cellular and chromosomal modules. In a similar experiment with onion bulbs hydroponically exposed to ZnO NMs at 20, 50, 75, and 100 mg L$^{-1}$, Kumari *et al.* (2011) concluded that the ZnO NMs were genotoxic and cytotoxic for the plants.

Hernandez-Viezcas *et al.* (2011) exposed velvet mesquite (*Prosopis juliflora- velutina*) plants for 15 days to ZnO NM at 0, 500 1000, 2000, and 4000 mg L$^{-1}$. The Zn concentrations and catalase activity increased in root, stems, and leaves at all concentrations, while the ascorbate peroxidase activity increased only in stems and leaves. These researchers also performed X-ray absorption near edge structure (XANES) and $\mu$-X-ray fluorescence ($\mu$-XRF) studies, revealing that ZnO was not found in any tissues in its nano-form, but in the form of Zn (II) with presence in the vascular system of roots and leaves, suggesting the biotransformation of ZnO NMs while being taken up by the plant.

Moving from hydroponic culture to a soil experiment, Du *et al.* (2011) completed a study in an agricultural field. They argued for the need to perform soil studies to mimic

realistic plant conditions. It is well known that ZnO NMs behave different in soil than they do in solution because after dissolution the released ions tend to interact with various components of the soil matrix. The authors concluded that ZnO NMs effectively dissolved in the soil, enhancing the plant uptake of Zn. The concentration used for this study was 5 g dispersed in approximately 36 kg of soil, where wheat (*Triticum aestivum* L.) plants were grown. Zinc content increased in stems and grains of treated plants, and soil enzyme activities were inhibited, pointing that ZnO NMs were toxic for the soil ecosystem.

In a short-time investigation by Shaymurat *et al.* (2012), the effects of ZnO NMs on root growth and genotoxicity were evaluated by a hydroponic exposure of garlic (*Allium sativum* L.) at 50 mg $L^{-1}$. The results showed a concentration-dependent blockage of the root growth, as well as nanoparticle-induced mitotic aberrations (such as chromosome stickiness, bridges and breakages), confirming genotoxic consequences of ZnO NMs.

Lee *et al.* (2012) performed an experiment to evaluate the soil microcosm with and without plants after soil contamination with ZnO NMs. They harvested buckwheat (*Fagopyrum esculentum*) plants after being exposed to 10, 100, and 1000 mg $kg^{-1}$ of ZnO NMs. The results indicated a concentration-dependent decrease in seedling growth and diminshed root length at 100 and 1000 mg $kg^{-1}$. Regarding the soil bacterial communities, the authors concluded that the toxic effects of ZnO NMs against bacteria might be diminished by the interaction between plants and soil.

Pokhrel & Dubey (2013) carried out a germination study by direct exposure of corn (*Zea mays* L.) and cabbage (*Brassica oleracea* var. capitata L.) to ZnO NMs and the dissolved ion $ZnSO_4$. The concentrations of 0.01, 0.1, 1, 10, 500, and 1000 $\mu$g $mL^{-1}$ were put in direct contact with the roots in petri dishes during seven days and showed the following effects: in corn, the primary root cells appeared elongated by the presence of ZnO NMs and the cells' shapes were distorted (of wider and shorter morphology) for the plants exposed to $ZnSO_4$ at 1000 $\mu$g $mL^{-1}$, compared to the control treatments. Furthermore, the same experiment (Pokhrel & Dubey, 2013) showed no effect on root elongation for the cabbage plants. However, there was a clear effect in germination for ZnO NMs and the

ionic compound. Seeds did not germinate at the highest concentration after exposure to $ZnSO_4$, and the seeds treated with ZnO NMs showed a 40% growth inhibition at the same concentration, in both cases, when compared to control plants.

Research on cowpea *(Vigna unguiculata)* plants grown in two standard soils and hydroponic solution compared the effects of ZnO NMs versus soluble $Zn^{2+}$. In this experiment, Wang *et al.* (2013) exposed cowpea plants to 500 mg $kg^{-1}$ of ZnO NMs and $ZnCl_2$ (or 25 mg Zn $L^{-1}$ in solution culture) and found $Zn^{2+}$ to be more toxic to the plants than the NMs, when grown in solution. Regarding the soil conditions, they did not find any differences in plant growth, Zn accumulation or speciation, after comparison between ZnO NMs and $ZnCl_2$. The authors concluded that ZnO NMs dissolved rapidly after their entry into the soil, however, they stated that this study did not present evidence of nanospecific risk.

Raliya *et al.* (2015) exposed soil-grown tomato (*Solanum lycopersicum* L.) plants to ZnO NMs through two different methods: foliar application and soil amendment. The evaluated concentrations include 0, 10, 100, 250, 500, 750, and 1000 mg $kg^{-1}$. ZnO NMs did not affect the germination. The plant height increased around 10% (amended soil), and the root length increased by 50% at 250 mg $kg^{-1}$ (foliar). The chlorophyll content increased at concentrations of 10 and 1000 mg $kg^{-1}$ for the soil amended with ZnO NMs, and the number of produced flowers increased in both exposure methods, after comparison with control plants. In both application methods the fruit yield was enhanced by 82% for foliar and 305% in the soil amendment. The authors concluded that NMs induced plant development when exposed to low concentrations; however, with large doses it declined. They also pointed to metal oxides as the possible cause for cytotoxicity.

A soil experiment by Bandyopadhyay *et al.* (2015) in symbiont alfalfa (*Medicago sativa-Sinorhizobium meliloti*) tested ZnO NMs, bulk ZnO, and ionic $ZnCl_2$ at concentrations ranging from 0 to 750 mg $kg^{-1}$. The authors found several parameters significantly reduced by the ionic Zn compound, including germination, root, and shoot biomass, catalase activity as well as total leaf protein. While ZnO NMs were found to affect only the germination at the highest concentrations, bulk ZnO reduced germination at 50% and increased root

and shoot biomass. The authors also reported accumulation of Zn in root tissues exposed to 500 mg $kg^{-1}$, which formed aggregates confirmed by scanning transmission electron microscopy (STEM) and energy-dispersive X-ray spectroscopy (EDX). From the results, they concluded that $ZnCl_2$ was more toxic than the ZnO NMs.

### 1.3.1 Studies in bean plants

Beans are the most consumed legume in the world due to its high protein content, as well as the good amounts of zinc and iron they provide at a low cost. In 2014, it was estimated a production over 26 million of tons world-wide (FAOSTAT, 2014). According to Jones (1999), beans are produced in a large range of environments and regions, such Latin America, Africa, the Middle East, China, Europe, the US, and Canada (see Figure 1.2).

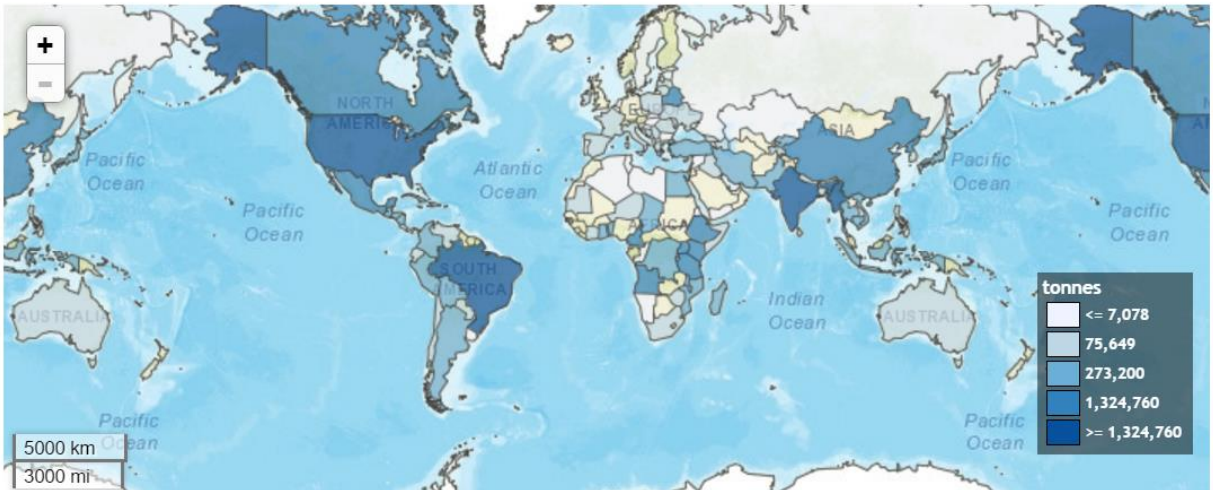


Figure 1.2: Dry bean production quantities by country during 2014. *Reproduced from FAOSTAT (2014)*

As a consequence of such a large production, there is a possibility that the ZnO NMs released into the environment could reach bean crops. Also, the wide consumption represents a possible link to humans via the food chain. Thus, the study of the effects of ZnO

NMs on bean plants represents a valuable tool to build more information about ZnO NMs' effects to the environment.

To the best of the author's knowledge there are no studies on the effects of Z-COTE and Z-COTE HP1 in common bean (*Phaseolus vulgaris* L.) plants. However, a recent study performed by García-Gómez *et al.* (2017) showed oxidative stress in bean plants induced by ZnO NMs (from Sigma-Aldrich), as well as changes in the photosynthetic pigment concentration. In addition, the authors suggested that the toxicity from the ZnO NMs was comparable to that of the bulk ZnO.

In the present doctoral research, the long-term exposure of Z-COTE and Z-COTE HP1 in common bean *(Phaseolus vulgaris)* was evaluated through the effects on plant growth, yield, nutritional quality of seeds, and residual effect of these NMs in seeds from second generation plants. The research was divided into three stages. Stage I was established to evaluate the effects of Z-COTE and Z-COTE HP1 in the plants physiological, biochemical, and agronomical parameters. Stage II was set to evaluate the effects of ZnO NMs in mature seeds harvested from two different soil types, while the residual effects of Z-COTE and Z-COTE HP1 in seeds from second generation plants were evaluated in stage III.

The **general objective** of this research work was to evaluate the effects of Z-COTE and Z-COTE HP1 on the development and yield of bean (*Phaseolus vulgaris* L. var. red hawk kidney) plants grown in two different types of soil.

The **specific objectives** were:

1. To evaluate the effects of ZnO NMs on the physiological development of bean plants grown in soil.

2. To quantify the Zn uptake by bean plant tissues after exposure to ZnO NMs.

3. To evaluate the impact on bean production of plants grown in soil amended with ZnO NMs.

4. To assess the nutritional composition of red kidney bean seeds produced by plants exposed to ZnO NMs.

5. To establish the effects of soil types in the uptake and translocation of ZnO NMs.

6. To determine the residual effects of ZnO NMs into a second generation of bean seeds.

This research was directed to prove the following hypotheses:

- Physiological parameters of bean plants are affected in a different manner when treated with Z-COTE and Z-COTE HP1, due to the specific surface properties of each NM.

- Plant uptake and translocation of Zn is altered in a distinct way after exposure to Z-COTE and Z-COTE HP1.

- Bean seeds' nutritional composition is affected by Z-COTE and Z-COTE HP1 in a different course.

- The interaction of Zn with soil organic matter modifies the uptake of Z-COTE and Z-COTE HP1 by bean plants.

- The residual effects of ZnO NMs on second generation plants impact the nutritional quality of the bean seeds.

In Stage I, bean plants were grown for 45 days in natural soil (NS) amended with either Z-COTE, Z-COTE-HP1, bulk ZnO, or ionic $ZnCl_2$ at 0, 62.5, 125, and 500 mg $kg^{-1}$. Growth parameters and essential elements were determined, in addition to Zn uptake and translocation to nodules, roots, stems, leaves, and pods. In Stage II, plant yield and seed nutrient composition were evaluated. Plants were grown to maturity in NS or organic matter-enriched soil (ES) amended with ZnO NMs, bulk ZnO or $ZnCl_2$. Seed yield along with macro and micronutrient composition was determined, and results were compared between the two different types of soil. Finally, in Stage III, seeds from plants cultivated in ES amended with 0-500 mg $kg^{-1}$ of the ZnO NMs were cultivated in ES without further exposure to ZnO NMs. The activity of antioxidant enzymes (catalase, ascorbate peroxidase,

and superoxide dismutase) was evaluated, and the nutritional quality of the seeds was assessed to identify residual effects caused by the early exposure to ZnO NMs.

Performing an evaluation of the exposure of Z-COTE and Z-COTE HP1 to beans (*Phaseolus vulgaris* L. var. red hawk kidney) provided experimental evidence of the potential effects of the use and disposal of commercially available ZnO NMs to edible plants. The outcomes of this investigation are a great input to build future regulations on the use and disposal of Z-COTE and Z-COTE HP1 NMs.

# Chapter 2

## Comparison of the effects of commercial coated and uncoated ZnO nanomaterials and Zn compounds in kidney bean *(Phaseolus vulgaris)* plants[1]

## 2.1 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 (Piccinno *et al.*, 2012). 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 (Peralta-Videa *et al.*, 2016, Yin *et al.*, 2015). 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 (Rizwan *et al.*, 2016). In addition, intentional use of nano-agricultural products represents a po-

[1]Reprinted from Medina-Velo, I. A., Barrios, A. C., Zuverza-Mena, N., Hernandez-Viezcas, J. A., Chang, C. H., Ji, Z., Zink, J. I., Peralta-Videa, J. R., and Gardea-Torresdey, J. L. (2017) Comparison of the effects of commercial coated and uncoated ZnO nanomaterials and Zn compounds in kidney bean *(Phaseolus vulgaris)* plants. *Journal of Hazardous Materials*, 332, 214-222. 

tential pathway for plant exposure to NMs (Rizwan *et al.*, 2016, Servin & White, 2016, Waalewijn-Kool *et al.*, 2014).

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 (Lin & Xing, 2007, Pokhrel & Dubey, 2013), root elongation (Ghodake *et al.*, 2011, Lin & Xing, 2007, Shaymurat *et al.*, 2012), genotoxicity and cytotoxicity (Kumari *et al.*, 2011), and Zn biotransformation (Hernandez-Viezcas *et al.*, 2011, Lopez-Moreno *et al.*, 2010). Soil studies revealed contradictory effects: negative impacts on plant growth (Bandyopadhyay *et al.*, 2015, Lee *et al.*, 2012, Yoon *et al.*, 2014) and yield (Zhao *et al.*, 2014), positive effects on growth (Mukherjee *et al.*, 2014, Raliya *et al.*, 2015), chlorophyll content, and fruit yield (Raliya *et al.*, 2015), and changes in the nutritional composition of plant tissues (Peralta-Videa *et al.*, 2014).

Zinc accumulation in plants exposed to ZnO NMs had shown concentration-dependent trends (Hernandez-Viezcas *et al.*, 2011, Mukherjee *et al.*, 2014, Peralta-Videa *et al.*, 2014, Priester *et al.*, 2012, Raliya *et al.*, 2015, Shaymurat *et al.*, 2012, Yoon *et al.*, 2014, Zhao *et al.*, 2014, 2015). 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 $ZnCl_2$ 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.

## 2.2 Materials and methods

### 2.2.1 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 (Yin *et al.*, 2015), 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 ≥99.0% purity was purchased from Sigma-Aldrich and $ZnCl_2$ ACS reagent ≥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.

### 2.2.2 Physicochemical characterization of ZnO NMs

The primary size and shape of the ZnO NMs were determined by transmission electron microscopy (TEM, FEI Tecnai T12, FEI, USA, accelerating voltage 80 kV), and samples were prepared by placing a drop of the ZnO suspension on copper grids, 400 mesh, carbon type-B at 50 $\mu$g $mL^{-1}$ in deionized water (DIW), followed by air drying at room temperature. Surface area was measured by the Brunauer Emmett and Teller (BET) equation using a Quantachrome Instrument (Quadrasorb SI, Quantachrome Instruments, USA) with nitrogen as the adsorption gas. Powder X-ray diffraction (XRD) spectra were obtained on a Philips diffractometer (X'Pert Pro, PANalytical, Netherlands), equipped with Cu K radiation for identifying the crystal structure of each material. Hydrodynamic size and zeta-potential measurements of the ZnO NMs suspensions in water were performed using a ZetaPALS instrument (Zeta Potential Analyzer, ZetaPALS, Brookhaven Instruments, USA). Stock solutions for the two types of ZnO were prepared at 1 mg $mL^{-1}$ by adding dried powder of nanomaterials in DIW. After these suspensions were sonicated for 15 min

in a water bath sonicator, stock solutions were diluted in DIW to provide 50 $\mu$g mL$^{-1}$ suspensions followed by further 15 min sonication to be used for size and zeta potential analysis. Material impurity was quantified using thermal gravimetric analysis (TGA) on a Pyris Diamond TG/DTA, Perkin Elmer and using inductively coupled plasma optical emission spectrometry (ICP-OES, ICPE-9000, Shimadzu, Japan).

### 2.2.3 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 $\pm$ 0.021, EC = 1705 $\pm$ 47.6 $\mu$S cm$^{-1}$, and TDS = 847.5 $\pm$ 23.8 mg L$^{-1}$).

Four replicates of the Z-COTE, Z-COTE HP1, bulk ZnO, and $ZnCl_2$ suspensions/solutions at 62.5, 125, 250 and 500 mg kg$^{-1}$ 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 DIW and bath sonicated (Crest Ultrasonics, Trenton, NJ) for 30 min at 25 °C and an intensity of 180 watts. $ZnCl_2$ 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% $HNO_3$) 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 $\mu$mole m$^{-2}$ s$^{-1}$), 25/20 °C day/night temperature and 65-70% relative humidity.

### 2.2.4 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 $HNO_3$ and deionized water (18 MΩ) alternately, and lengths were measured. For leaf length, measurement was taken along the central vain 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.

### 2.2.5 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 *et al.* (2014).

### 2.2.6 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 Tukeys HSD test at a p-value of 0.05. Data presented are means ± standard errors (SE) of specified number of replicates.

## 2.3 Results and discussion

### 2.3.1 Physicochemical characterization of ZnO NMs

As it can observed on Table 2.1 and Figure 2.1 both coated and uncoated ZnO NMs had similar primary sizes in the range of 10-300 nm with elongated morphologies as determined by TEM (Figures S1a and b). The mean diameter is very similar ($d_{mean}$ = 93.8 and 84.1 nm for Z-COTE and Z-COTE HP1, respectively) and a large number of nanoparticles (>95%) stayed in the similar range of 10-150 nm, as shown in Figures 2.1b and c. In the same manner, hydrodynamic diameter showed a high monodispersion in DI water (PdI = 0.167 and 0.165, respectively). XRD analysis demonstrated that the entire material phase was hexagonal (JCPDS 03-065-3411) without showing diffraction peaks of any impurities. Z-COTE and Z-COTE HP1 showed similarities in the surface area (13-17 $m^2$ $g^{-1}$) and hydrodynamic diameters (276-286 nm). Uncoated Z-COTE carries a positive zeta potential of 21.8 ± 0.8 mV, while the hydrophobic coated Z-COTE HP1 has a negative charge of -23.6 ± 0.9 mV in deionized water, likely due to the coating of triethoxycaprylylsilane. TGA and ICP analysis suggested a Z-COTE purity higher than 99% and Z-COTE HP1 has between 1 and 2% surface coating of triethoxycaprylylsilane.

Table 2.1: Physicochemical characterization summary of the ZnO nanomaterials

| Property | Technique | Units | ZnO NM | |
|---|---|---|---|---|
| | | | Z-COTE | Z-COTE HP1 |
| Primary size | TEM | nm | 10-300 | 10-300 |
| Phase and structure | XRD | | Hexagonal | Hexagonal |
| Shape/Morphology | TEM | | Elongated shape | Elongated shape |
| Surface area | BET | $m^2$ $g^{-1}$ | 16.6 | 13.1 |
| Size in DI water (50 $\mu$g $mL^{-1}$) | ZetaPALS | nm | 286 ± 2 | 276 ± 7 |
| Zeta potential in DI water | ZetaPALS | mV | 21.8 ± 0.8 | -23.6 ± 0.9 |
| Purity | ICP-OES TGA | wt(%) | 99.1 ± 0.2 | 98.2 ± 0.6 |
| | TGA | wt(%) | >99 | 98.7 |

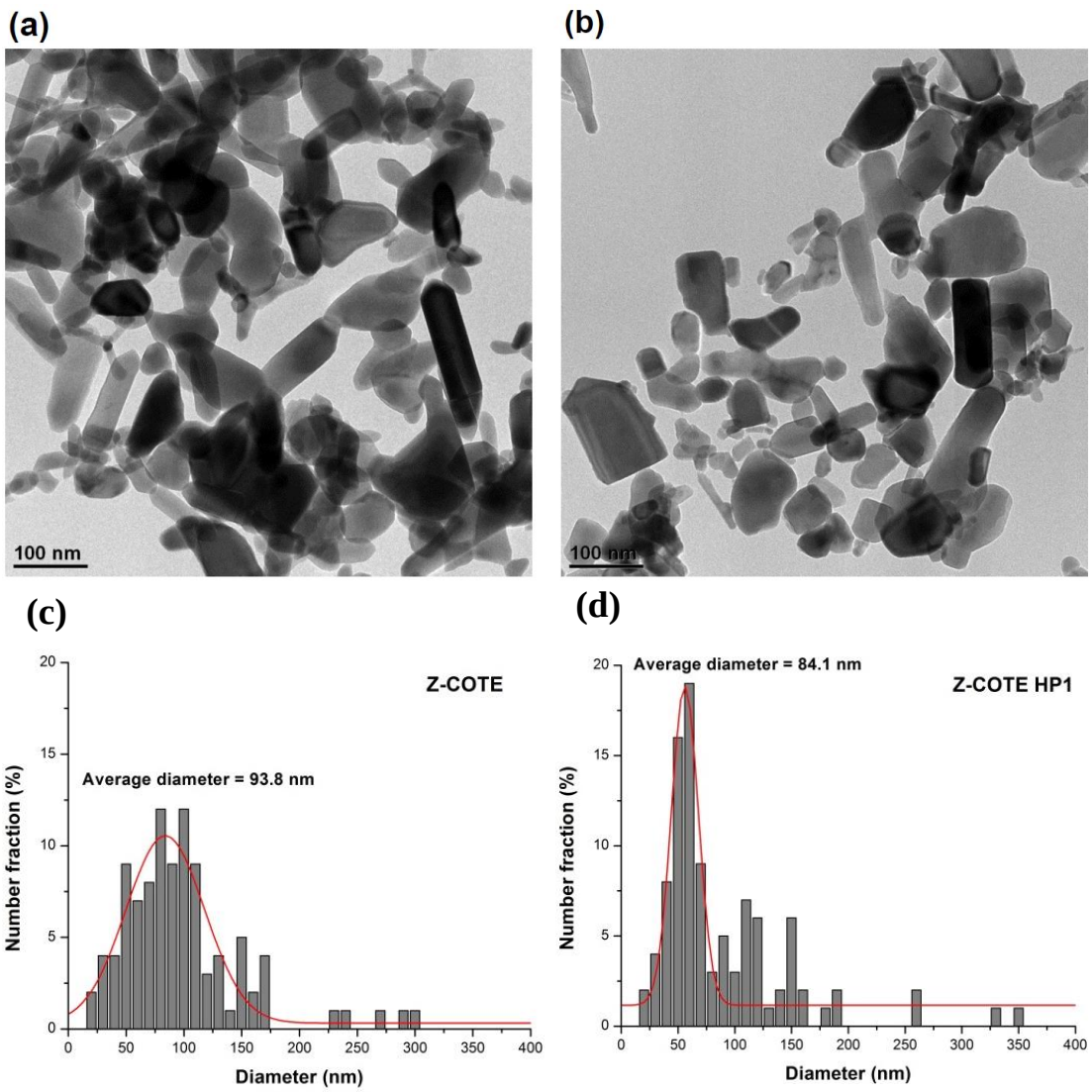


Figure 2.1: TEM images of (a) Z-COTE and (b) Z-COTE HP1. Primary size distribution of (c) Z-COTE and (d) Z-COTE HP1 by TEM

### 2.3.2 Seed germination and pod production

Table 2.2 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 2.2, none

of the nanomaterials/compounds show significant differences in seed germination and pod production, compared to controls. Furthermore, seed production was not compromised.

Table 2.2: Germination and number of pods produced by red kidney bean plants exposed to Z-COTE, Z-COTE HP1, bulk ZnO, $ZnCl_2$ and DI water (control) at concentrations of 62.5, 125, 250 and 500 mg $kg^{-1}$ of soil. Data are mean ± standard error of treatments (n=16 for controls, n=4 for Zn compounds) Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$).

| | | Number of plants/pods ± standard error | | | |
|---|---|---|---|---|---|
| | | **62.5 mg $kg^{-1}$** | **125 mg $kg^{-1}$** | **250 mg $kg^{-1}$** | **500 mg $kg^{-1}$** |
| **Germination** | *Control* | 4.8 ± 0.1 *a* | 4.8 ± 0.1 *a* | 4.8 ± 0.1 *a* | 4.8 ± 0.1 *a* |
| **(number of plants)** | *Bulk* | 5.0 ± 0.0 *a* | 5.0 ± 0.0 *a* | 4.5 ± 0.5 *a* | 4.8 ± 0.3 *a* |
| | *Z-COTE* | 4.8 ± 0.3 *a* | 4.5 ± 0.3 *a* | 4.5 ± 0.3 *a* | 4.8 ± 0.3 *a* |
| | *Z-COTE HP1* | 5.0 ± 0.0 *a* | 4.8 ± 0.3 *a* | 5.0 ± 0.0 *a* | 5.0 ± 0.0 *a* |
| | *$ZnCl_2$* | 4.8 ± 0.3 *a* | 4.5 ± 0.5 *a* | 4.8 ± 0.3 *a* | 5.0 ± 0.0 *a* |
| **Immature pods** | *Control* | 8.0 ± 1.2 *a* | 8.0 ± 1.2 *a* | 8.0 ± 1.2 *a* | 8.0 ± 1.2 *a* |
| **(number)** | *Bulk* | 8.3 ± 2.3 *a* | 6.8 ± 1.3 *a* | 8.3 ± 2.4 *a* | 8.0 ± 2.0 *a* |
| | *Z-COTE* | 5.3 ± 1.8 *a* | 6.8 ± 0.6 *a* | 4.8 ± 1.4 *a* | 5.8 ± 2.8 *a* |
| | *Z-COTE HP1* | 7.5 ± 2.2 *a* | 6.3 ± 1.7 *a* | 9.5 ± 1.7 *a* | 4.8 ± 1.1 *a* |
| | *$ZnCl_2$* | 2.8 ± 0.6 *a* | 2.8 ± 0.9 *a* | 4.0 ± 1.3 *a* | 2.8 ± 0.8 *a* |
| **Mature pods** | *Control* | 8.2 ± 0.4 *a* | 8.2 ± 0.4 *a* | 8.2 ± 0.4 *a* | 8.2 ± 0.4 *a* |
| **(number)** | *Bulk* | 8.3 ± 1.3 *a* | 9.8 ± 0.9 *a* | 7.5 ± 1.0 *a* | 7.8 ± 0.9 *a* |
| | *Z-COTE* | 8.8 ± 0.8 *a* | 9.3 ± 1.7 *a* | 10.5 ± 1.0 *a* | 10.0 ± 1.8 *a* |
| | *Z-COTE HP1* | 9.8 ± 0.3 *a* | 10.3 ± 0.9 *a* | 11.0 ± 1.2 *a* | 9.8 ± 0.6 *a* |
| | *$ZnCl_2$* | 9.8 ± 1.1 *a* | 9.1 ± 0.8 *a* | 9.5 ± 1.0 *a* | 8.5 ± 0.9 *a* |

### 2.3.3 Effects on plant growth

Figure 2.2 displays phenotypical changes in bean plants exposed to the different Zn compounds. As seen in Figure 2.2a, at 62.5 mg $kg^{-1}$, 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 $kg^{-1}$ (Bandyopadhyay *et al.*, 2015), as well as in tomato root length exposed to ZnO NM at 250 mg $kg^{-1}$ and above (Raliya *et al.*, 2015) and buckwheat roots at 100 mg $kg^{-1}$ (Lee *et al.*, 2012). However, other studies have shown contrary results. ZnO NM at 500 mg $kg^{-1}$, increased soybean root length (Lopez-Moreno *et al.*, 2010), and at 0-500 mg $kg^{-1}$, green pea root length (Mukherjee *et al.*, 2014). 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 (Epstein, 1994).

None of the NMs affected stem elongation (Figure 2.2b); however, bulk ZnO, at 250 and 500 mg $kg^{-1}$, increased stem length by 30% and 31%, while $ZnCl_2$ at 500 mg $kg^{-1}$ reduced it by 25%, compared with control. Similar results have been reported in alfalfa (Bandyopadhyay *et al.*, 2015) and in green pea (Mukherjee *et al.*, 2014). Mukherjee *et al.* (2014) associated the shoot length promotion in green pea with the low oxidative radicals produced by bulk ZnO, and the toxicity of the $ZnCl_2$, with the high Zn accumulation in tissues, which was corroborated in the present study (Figure 2.4).

Figure 2.2c 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 $kg^{-1}$ and bulk ZnO at 125 mg $kg^{-1}$ and above, increased leaf length (up to 24%) ($p \leq 0.05$). Oppositely, $ZnCl_2$ shortened leaf length by 24%, 20%, and 32% at 62.5, 250, and 500 mg $kg^{-1}$, respectively. The effects of Z-COTE HP1 could be produced by Si in the surface coating (Epstein, 1994), and the effect of bulk ZnO could be attributed to a lower production in reactive oxygen species (Mukherjee *et al.*, 2014). Negative effects of $ZnCl_2$ might be attributed to an excess of chloride in leaves, which has shown to decrease leaf size in beans (Novikova *et al.*, 2014).

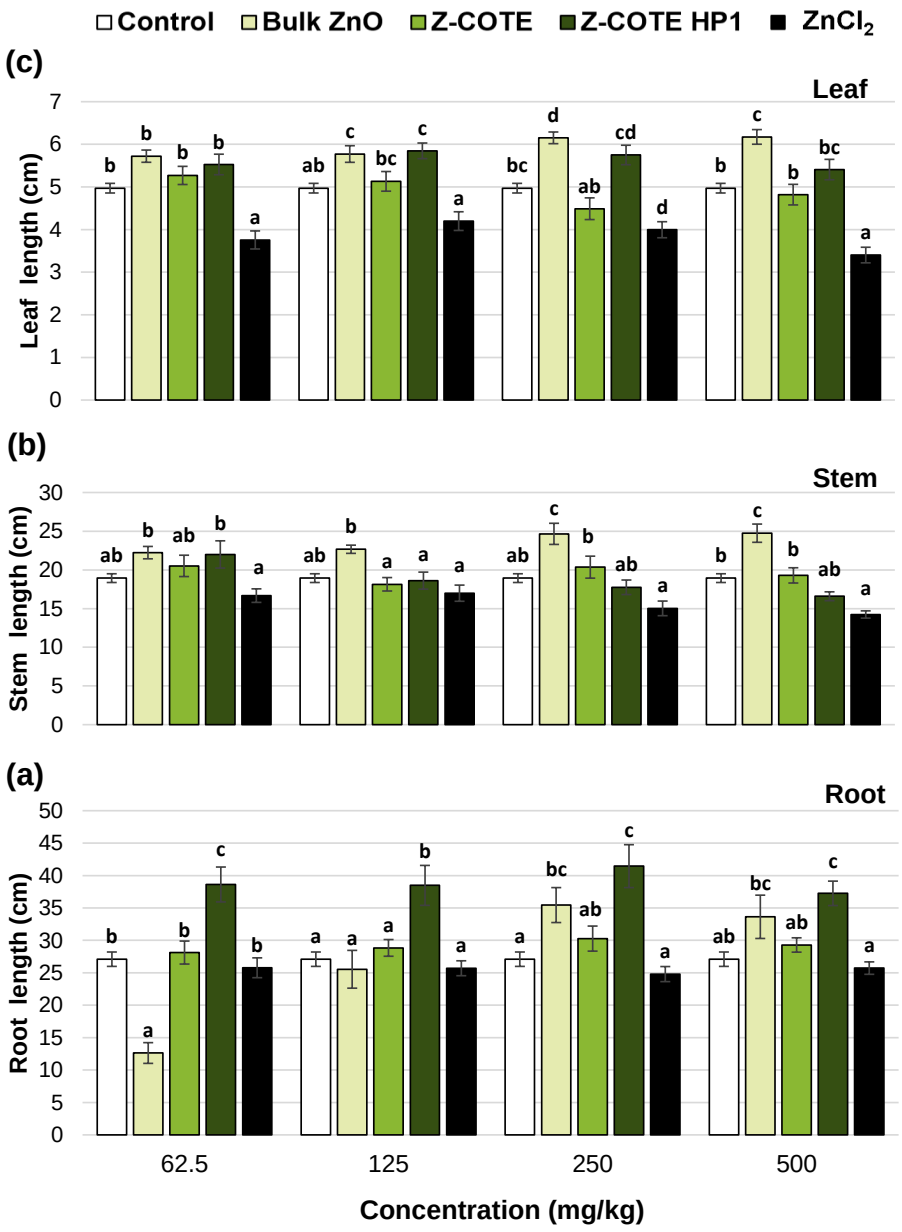


Figure 2.2: Length of (a) root, (b) stem, and (c) leaf tissues of red kidney bean plants exposed to Z-COTE, Z-COTE HP1, bulk ZnO, and $ZnCl_2$ at 0 (control), 62.5, 125, 250 and 500 mg $kg^{-1}$ of soil. Values are means ± SE of 4 replicates for Zn compounds and 16 for controls. Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$).

### 2.3.4 Biomass production

The biomass data is shown in Table 2.3. 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 $kg^{-1}$ 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 (Novikova *et al.*, 2014). Additionally, $ZnCl_2$ at 500 mg $kg^{-1}$ 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 (Hajrasuliha, 1980, Kabata-Pendias, 2011, Tavakkoli *et al.*, 2010). Chloride has shown toxicity to photosystem II, which affects the whole plant system (Tavakkoli *et al.*, 2010). $ZnCl_2$ significantly reduced alfalfa biomass (Bandyopadhyay *et al.*, 2015). In addition, Wang *et al.* (2013) reported that $ZnCl_2$ was more toxic to cowpea than ZnO NMs.

### 2.3.5 Effects of treatments on relative chlorophyll content in leaves

Figure 2.3 shows the relative chlorophyll content of kidney bean plants exposed to the Zn NMs or compounds. Opposite to Mukherjee *et al.* (2014) 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, $ZnCl_2$ at 250 and 500 mg $kg^{-1}$ decreased relative chlorophyll content by 66 and 54%, with respect to control ($p \leq 0.05$). Chloroplasts are highly permeable to $Cl^-$ (Heber & Heldt, 1981), which reduces the photosynthetic capacity as a result of chlorophyll degradation caused by an impact in photosystem II (Hajrasuliha, 1980, Tavakkoli *et al.*, 2010).

Table 2.3: Fresh weight, dry weight and water content of roots, stems, leaves, and nodules of red kidney bean plants exposed to Z-COTE, Z-COTE HP1, bulk ZnO, $ZnCl_2$ and DI water (control) at concentrations of 62.5, 125, 250 and 500 mg $kg^{-1}$ of soil. Data are mean ± standard error of treatments (n=16 for controls, n=4 for Zn compounds). Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$).

| | | | Weight of tissues (mg) ± standard error | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **62.5 mg $kg^{-1}$** | | **125 mg $kg^{-1}$** | | **250 mg $kg^{-1}$** | | **500 mg $kg^{-1}$** | |
| ***Roots*** | **Fresh weight (mg)** | *Control* | 10934 ± 1006 | *ab* | 10934 ± 1006 | *ab* | 10934 ± 1006 | *ab* | 10934 ± 1006 | *ab* |
| | | *Bulk* | 6035 ± 854 | *a* | 5644 ± 782 | *ab* | 4962 ± 253 | *a* | 6149 ± 688 | *a* |
| | | *Z-COTE* | 11260 ± 1498 | *ab* | 11887 ± 465 | *b* | 11107 ± 642 | *ab* | 9575 ± 906 | *ab* |
| | | *Z-COTE HP1* | 17456 ± 663 | *b* | 16818 ± 527 | *b* | 16532 ± 1105 | *b* | 13251 ± 1249 | *b* |
| | | *$ZnCl_2$* | 11339 ± 800 | *ab* | 11355 ± 734 | *b* | 10991 ± 763 | *b* | 10272 ± 638 | *ab* |
| | **Dry weight (mg)** | *Control* | 1170 ± 60 | *a* | 1170 ± 60 | *a* | 1170 ± 60 | *b* | 1170 ± 60 | *a* |
| | | *Bulk* | 900 ± 89 | *a* | 1047 ± 62 | *a* | 714 ± 38 | *a* | 957 ± 35 | *a* |
| | | *Z-COTE* | 1248 ± 63 | *a* | 1201 ± 36 | *a* | 1134 ± 36 | *b* | 1134 ± 65 | *a* |
| | | *Z-COTE HP1* | 1331 ± 105 | *a* | 1325 ± 30 | *a* | 1418 ± 99 | *b* | 1306 ± 74 | *a* |
| | | *$ZnCl_2$* | 1264 ± 185 | *a* | 1020 ± 71 | *a* | 1127 ± 150 | *ab* | 1003 ± 66 | *a* |
| ***Stems*** | **Fresh weight (mg)** | *Control* | 8977 ± 416 | *ab* | 8977 ± 416 | *ab* | 8977 ± 416 | *a* | 8977 ± 416 | *a* |
| | | *Bulk* | 10545 ± 471 | *b* | 10226 ± 742 | *b* | 8580 ± 809 | *a* | 9735 ± 486 | *a* |
| | | *Z-COTE* | 8645 ± 510 | *ab* | 8287 ± 373 | *ab* | 8688 ± 759 | *a* | 7726 ± 922 | *a* |
| | | *Z-COTE HP1* | 9974 ± 710 | *ab* | 8238 ± 184 | *ab* | 9552 ± 483 | *a* | 8372 ± 238 | *a* |
| | | *$ZnCl_2$* | 7317 ± 533 | *a* | 7313 ± 562 | *b* | 7925 ± 346 | *a* | 7359 ± 402 | *a* |
| | **Dry weight (mg)** | *Control* | 1476 ± 78 | *a* | 1476 ± 78 | *a* | 1476 ± 78 | *ab* | 1476 ± 78 | *b* |
| | | *Bulk* | 1436 ± 106 | *a* | 1502 ± 168 | *a* | 1177 ± 108 | *a* | 1769 ± 85 | *b* |
| | | *Z-COTE* | 1587 ± 89 | *a* | 1496 ± 125 | *a* | 1636 ± 186 | *ab* | 1443 ± 165 | *b* |
| | | *Z-COTE HP1* | 1825 ± 181 | *a* | 1569 ± 25 | *a* | 1909 ± 198 | *b* | 1449 ± 46 | *b* |
| | | *$ZnCl_2$* | 1355 ± 139 | *a* | 1198 ± 78 | *a* | 1272 ± 126 | *a* | 902 ± 60 | *a* |
| ***Leaves*** | **Fresh weight (mg)** | *Control* | 10708 ± 833 | *ab* | 10708 ± 833 | *ab* | 10708 ± 833 | *ab* | 10708 ± 833 | *bc* |
| | | *Bulk* | 13075 ± 247 | *b* | 12755 ± 650 | *ab* | 12019 ± 901 | *b* | 13103 ± 766 | *c* |
| | | *Z-COTE* | 9851 ± 1046 | *ab* | 11073 ± 687 | *ab* | 10519 ± 1729 | *ab* | 7597 ± 505 | *ab* |
| | | *Z-COTE HP1* | 12610 ± 1499 | *b* | 12881 ± 582 | *b* | 12596 ± 1135 | *b* | 11437 ± 113 | *bc* |
| | | *$ZnCl_2$* | 6106 ± 676 | *a* | 7889 ± 437 | *a* | 5750 ± 1007 | *a* | 4865 ± 469 | *a* |
| | **Dry weight (mg)** | *Control* | 1351 ± 92 | *ab* | 1351 ± 92 | *ab* | 1351 ± 92 | *ab* | 1351 ± 92 | *b* |
| | | *Bulk* | 1576 ± 108 | *b* | 1539 ± 56 | *abc* | 1233 ± 91 | *ab* | 1809 ± 137 | *b* |
| | | *Z-COTE* | 1474 ± 146 | *ab* | 1729 ± 143 | *bc* | 1785 ± 304 | *b* | 1241 ± 109 | *b* |
| | | *Z-COTE HP1* | 1628 ± 228 | *b* | 1987 ± 143 | *c* | 1820 ± 149 | *b* | 1786 ± 53 | *b* |
| | | *$ZnCl_2$* | 856 ± 95 | *a* | 1069 ± 60 | *a* | 677 ± 154 | *a* | 582 ± 79 | *a* |
| ***Nodules*** | **Fresh weight (mg)** | *Control* | 677 ± 86 | *ab* | 677 ± 86 | *ab* | 677 ± 86 | *ab* | 677 ± 86 | *a* |
| | | *Bulk* | 318 ± 205 | *a* | 347 ± 82 | *a* | 245 ± 131 | *a* | 292 ± 76 | *a* |
| | | *Z-COTE* | 773 ± 149 | *ab* | 970 ± 160 | *b* | 956 ± 182 | *b* | 531 ± 107 | *a* |
| | | *Z-COTE HP1* | 1065 ± 153 | *b* | 1147 ± 119 | *b* | 977 ± 68 | *b* | 753 ± 121 | *a* |
| | | *$ZnCl_2$* | 670 ± 174 | *ab* | 681 ± 75 | *ab* | 540 ± 216 | *ab* | 323 ± 55 | *a* |
| | **Dry weight (mg)** | *Control* | 89 ± 11 | *a* | 89 ± 11 | *ab* | 89 ± 11 | *a* | 89 ± 11 | *a* |
| | | *Bulk* | 56 ± 42 | *a* | 52 ± 6 | *a* | 50 ± 26 | *a* | 85 ± 28 | *a* |
| | | *Z-COTE* | 89 ± 19 | *a* | 93 ± 14 | *ab* | 85 ± 13 | *a* | 70 ± 10 | *a* |
| | | *Z-COTE HP1* | 134 ± 22 | *a* | 138 ± 13 | *b* | 112 ± 24 | *a* | 72 ± 10 | *a* |
| | | *$ZnCl_2$* | 89 ± 32 | *a* | 69 ± 8 | *a* | 58 ± 22 | *a* | 34 ± 6 | *a* |

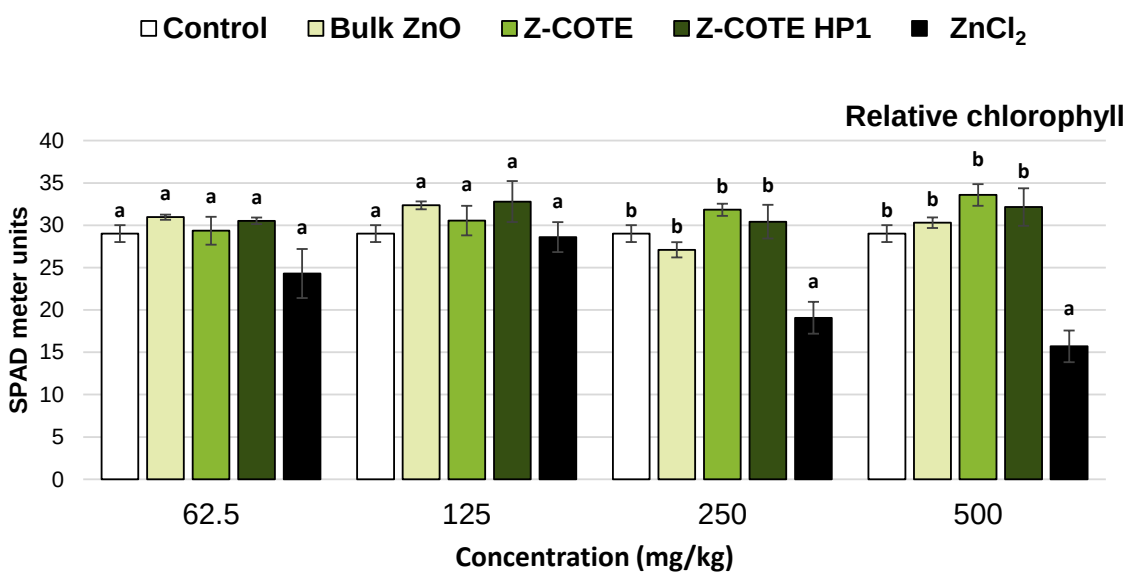


Figure 2.3: Relative chlorophyll content of bean leaves exposed to Z-COTE, Z-COTE HP1, bulk ZnO, and $ZnCl_2$ at 0 (control), 62.5, 125, 250 and 500 mg $kg^{-1}$ of soil. Values are means ± SE of 4 replicates for Zn compounds and 16 for controls. Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$).

### 2.3.6 Zinc uptake and translocation

Similarly to previous studies (Hernandez-Viezcas *et al.*, 2011, Mukherjee *et al.*, 2014, Peralta-Videa *et al.*, 2014, Priester *et al.*, 2012, Raliya *et al.*, 2015, Shaymurat *et al.*, 2012, Yoon *et al.*, 2014, Zhao *et al.*, 2014, 2015), kidney bean plants accumulated Zn in a concentration-dependent manner, after exposure to Z-COTE and Z-COTE HP1 at concentrations of 125 mg $kg^{-1}$ and above (Figure 2.4 ). As expected, there was higher Zn accumulation in the root system.

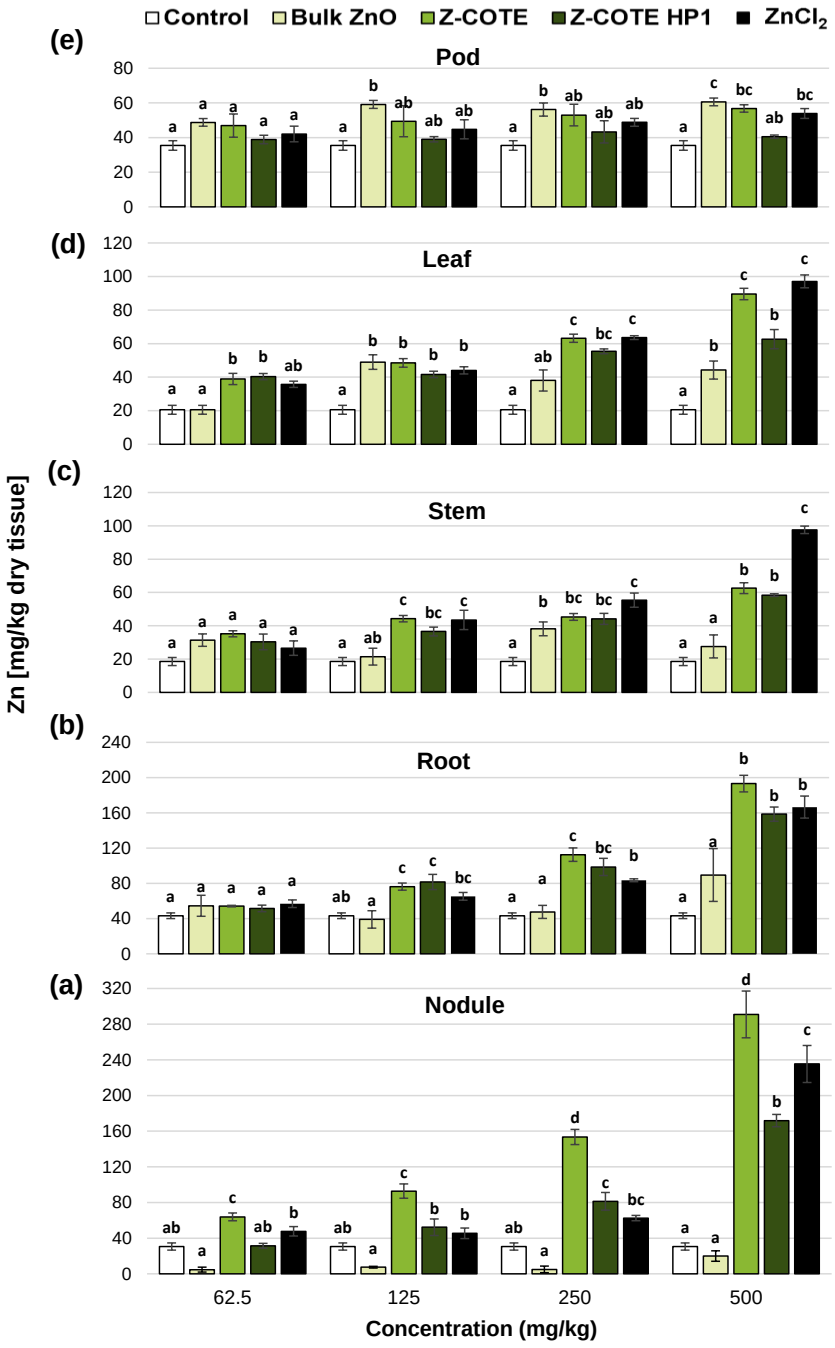


Figure 2.4: Zinc uptake of (a)nodules, (b)roots, (c)stems, (d)leaves, and (e)pods of bean plants exposed to Z-COTE, Z-COTE HP1, bulk ZnO, and $ZnCl_2$ at 0 (control), 62.5, 125, 250 and 500 mg $kg^{-1}$ of soil. Values are means ± SE of 4 replicates for Zn compounds and 16 for controls. Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$).

All roots exposed to Z-COTE had more Zn in nodules ($p \leq 0.05$), compared with control and the other treatments (Figure 2.4a). Increases in nodule Zn under Z-COTE varied from 109% at 62.5 mg $kg^{-1}$ to 850% at 500 mg $kg^{-1}$, followed by Z-COTE HP1 at 250 and 500 mg $kg^{-1}$ (166% and 461%, respectively), and $ZnCl_2$ at 500 mg $kg^{-1}$ (669%), compared with control plants (Figure 2.4a). None of the bulk ZnO concentrations increased Zn in nodules, compared with control. This could be an effect of particle size (<1000 nm, (Dimkpa *et al.*, 2012)) and aggregation.

In the root system, none of the treatments at the 62.5 mg $kg^{-1}$ affected Zn accumulation (Figure 2.4b); very likely due to fixation of Zn in the soil matrix (Zhao *et al.*, 2012). At 125 mg $kg^{-1}$ 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 (Dimkpa *et al.*, 2012). Zn accumulation in roots from ZnO NMs and $ZnCl_2$ showed different tendency at 125 mg $kg^{-1}$ than at 250 and 500 mg $kg^{-1}$. However, the statistical comparisons of treatment means were clearly defined only at the highest concentration. At 500 mg $kg^{-1}$, root Zn was similar for ZnO NMs and $ZnCl_2$ (Figure 2.4b). 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 (Wang *et al.*, 2010, Zhao *et al.*, 2013b). In. addition,Wu *et al.* (2010) 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 2.4c). However, at the highest treatment concentration (500 mg $kg^{-1}$) the stem Zn in plants exposed to $ZnCl_2$ 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 (Guerinot, 2000).

The Zn accumulation in leaves was similar to that in stems, except at 62.5 mg $kg^{-1}$, where accumulation from Z-COTE, Z-COTE HP1 and ($p \leq 0.05$) was significantly higher (89, 96, and 74%, respectively), compared with control ($p \leq 0.05$) (Figure 2.4d). Higher leaf Zn was found at 500 mg $kg^{-1}$ 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 $ZnCl_2$ (Bandyopadhyay *et al.*, 2015).

Of particular interest is the Zn accumulation in pods (Figure 2.4e). At 62.5 mg $kg^{-1}$, none of the NMs or compounds reduced Zn accumulation in pods. Conversely, plant exposure to bulk ZnO at 125 mg $kg^{-1}$ and above, resulted in a significant increase in pod Zn, compared with control ($p \leq 0.05$). Increases ranged from 67% at 125 mg $kg^{-1}$ to 71% at 500 mg $kg^{-1}$. On the other hand, under exposure to the nanoparticulate forms, pod Zn increased, significantly, only at 500 mg $kg^{-1}$ of uncoated Z-COTE. Results suggest that none of the compounds downregulated the genes involved in Zn transport associated genes in developing pods (Astudillo-Reyes *et al.*, 2015).

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

#### Effects in macronutrient composition

Accumulation of macroelements altered by Zn treatments is shown in Table 2.4. At 62.5 mg $kg^{-1}$, 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 $ZnCl_2$.

Coated Z-COTE HP1 at 125 and 500 mg $kg^{-1}$, 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 $\pm$ 0.9 mV), favoring a simultaneous uptake (Gransee & Führs, 2013). Sulfur, which is available to plants in the form of anionic sulfate ($SO_4^{2-}$)(Leustek & Saito, 1999), was augmented 64.7% in roots exposed to 125 mg $kg^{-1}$

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 (Leustek & Saito, 1999), increasing the S uptake. The fact that only the 125 mg $kg^{-1}$ 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 (Kataoka *et al.*, 2004).

Bulk ZnO significantly reduced Ca in stems and leaves, compared with control. In stems, Ca was reduced 42.3% at 500 mg $kg^{-1}$; in leaves, 29.7% at 250 and 28.5% at 500 mg $kg^{-1}$. Magnesium was also diminished in leaves of plants exposed to bulk ZnO at 62.5 and 250 mg $kg^{-1}$ (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 (Guo *et al.*, 2016); 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 (Steucek & Koontz, 1970).

$ZnCl_2$, at the highest concentration increased Ca in roots and stems by 32.0% and 52.7% respectively, compared with control. According to Hajrasuliha (1980), $Cl^-$ 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 (Guo *et al.*, 2010); thus $ZnCl_2$ affected the accumulation of both ions. In addition, $Cl^-$ acts as a $Mg^{2+}$ accompanying anion; thus, they might be taken up together (Gransee & Führs, 2013). Finally, $ZnCl_2$ at 500 mg $kg^{-1}$, 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.

Table 2.4: Macronutrients of roots, stems and leaves of bean plants altered after exposure to Z-COTE, Z-COTE HP1, bulk ZnO, and $ZnCl_2$ at 0 (control), 62.5, 125, 250 and 500 mg $kg^{-1}$ of soil. Values are means ± 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 the nutrient concentration, compared to the respective control ($p \leq 0.05$).

| | | | **Concentration (mg $kg^{-1}$ dry tissue)** | | | |
|---|---|---|---|---|---|---|
| **Tissue** | **Nutrient** | **Treatment** | **62.5 mg $kg^{-1}$** | **125 mg $kg^{-1}$** | **250 mg $kg^{-1}$** | **500 mg $kg^{-1}$** |
| **Leaf** | Ca | Control | 29606 ± 1044 ab | 29606 ± 1044 ab | 29606 ± 1044 b | 29606 ± 1044 bc |
| | | ZnO Bulk | 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 |
| | | ZnO Bulk | 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 |
| | | ZnO Bulk | 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 |
| | | ZnO Bulk | 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 |
| | | ZnO Bulk | 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 |
| | | ZnO Bulk | 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 |
| | | ZnO Bulk | 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 |
| | | ZnO Bulk | 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 |

**Effects in micronutrient accumulation**

Table 2.5 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 $kg^{-1}$ reduced Mo uptake by 95%, Z-COTE HP1 increased B by 114% (250 mg $kg^{-1}$) and Mo by 76% (500 mg $kg^{-1}$); and Z-COTE at 250 mg $kg^{-1}$ increased B by 121%, compared with control.

Molybdenum is taken by plants as $MoO_4^{2-}$ (Nie *et al.*, 2014); however, it forms $ZnMoO_4$ with Zn ions, which is insoluble in water (Karekar *et al.*, 2015); 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 triethoxycaprylylsilane upregulating the MOT1 transporter in the root cell plasma membrane, increasing Mo uptake (Tomatsu *et al.*, 2007). 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 $\pm$ 0.9 mV) that may attract B, enhancing its uptake (Barrios *et al.*, 2017). 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 (Conde *et al.*, 2010).

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% at125 and 250 mg $kg^{-1}$, respectively, while Mn was 74% higher (at 125 mg $kg^{-1}$) than control. B is absorbed from the soil following the water flow through the roots (Hu & Brown, 1997), and then it is transported to the xylem by a facilitating boron transporter 1 (BOR1) (Miwa & Fujiwara, 2010) 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 trans-

port, including members of the ZIP (zinc-regulated transporter/iron-regulated transporter (ZRT/IRT1)-related protein) transporter family (Pittman, 2005). 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 $kg^{-1}$, 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 (Millaleo *et al.*, 2010) 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 $kg^{-1}$, respectively, compared with control. It has been reported that high levels of Zn can reduce Cu uptake, by an antagonist interaction (Kabata-Pendias, 2011), due to both elements sharing the same transporters (Alloway, 2004).

At 250 and 500 mg $kg^{-1}$, 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 (Mousavi *et al.*, 2012); thus, it is hypothesized that the reduction in stem B was due to an antagonistic effect between B and Zn at translocation level.

Table 2.5: Micronutrients of roots, stems and leaves of red kidney bean plants altered after exposure to Z-COTE, Z-COTE HP1, bulk ZnO, and $ZnCl_2$ at 0 (control), 62.5, 125, 250 and 500 mg $kg^{-1}$ of soil. Values are means ± 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 the nutrient concentration, compared to the respective control ($p \leq 0.05$).

| | | **Treatment** | | **Nutrient concentration** | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Tissue** | **Nutrient** | ***Compound*** | ***mg kg*$^{-1}$** | ***mg kg*$^{-1}$ *dry tissue*** | ± | ***Std. error*** | **% change** | | |
| **Leaf** | Mn | Control | *** | 98.6 | ± | 4.9 | 100 | % | |
| | | ZnO Bulk | 62.5 | 57.8 | ± | 3.8 | 41 | % | - |
| | | | 125 | 54.9 | ± | 3.1 | 44 | % | - |
| | | | 250 | 47.1 | ± | 14.7 | 52 | % | - |
| | | | 500 | 53.3 | ± | 14.4 | 46 | % | - |
| | | $ZnCl_2$ | 500 | 128.9 | ± | 13.5 | 31 | % | + |
| | Fe | Control | *** | 58.3 | ± | 2.7 | 100 | % | |
| | | $ZnCl_2$ | 500 | 94.0 | ± | 18.3 | 61 | % | + |
| | Cu | Control | *** | 6.5 | ± | 0.3 | 100 | % | |
| | | ZnO Bulk | 250 | 4.4 | ± | 0.2 | 32 | % | - |
| | | | 500 | 3.9 | ± | 0.3 | 40 | % | - |
| | | $ZnCl_2$ | 500 | 8.7 | ± | 0.6 | 34 | % | + |
| **Stem** | Fe | Control | *** | 54.6 | ± | 3.6 | 100 | % | |
| | | $ZnCl_2$ | 500 | 104.8 | ± | 21.3 | 92 | % | + |
| | Mo | Control | *** | 51.2 | ± | 3.8 | 100 | % | |
| | | ZnO Bulk | 500 | 77.8 | ± | 10.1 | 52 | % | + |
| | | $ZnCl_2$ | 500 | 98.3 | ± | 9.0 | 92 | % | + |
| | B | Control | *** | 21.6 | ± | 2.2 | 100 | % | |
| | | ZnO Bulk | 250 | 4.1 | ± | 0.1 | 81 | % | - |
| | | | 500 | 1.9 | ± | 0.3 | 91 | % | - |
| | | Z-COTE | 125 | 48 | ± | 15.3 | 122 | % | + |
| | | | 250 | 46.6 | ± | 2.5 | 116 | % | + |
| | Mn | Control | *** | 14.6 | ± | 1.3 | 100 | % | |
| | | ZnO Bulk | 250 | 5.5 | ± | 0.7 | 62 | % | - |
| | | | 500 | 7.7 | ± | 0.3 | 47 | % | - |
| | | Z-COTE | 125 | 25.4 | ± | 1.1 | 74 | % | + |
| | Cu | Control | *** | 5.2 | ± | 0.3 | 100 | % | |
| | | ZnO Bulk | 62.5 | 2.8 | ± | 0.4 | 46 | % | - |
| | | | 125 | 1.9 | ± | 0.2 | 63 | % | - |
| | | | 250 | 2.8 | ± | 0.3 | 46 | % | - |
| | | | 500 | 1.9 | ± | 0.1 | 63 | % | - |
| | | $ZnCl_2$ | 500 | 6.8 | ± | 0.3 | 31 | % | + |
| | Ni | Control | *** | 1.5 | ± | 0.2 | 100 | % | |
| | | ZnO Bulk | 62.5 | 0.6 | ± | 0.2 | 60 | % | - |
| | | | 500 | 0.2 | ± | 0.2 | 87 | % | - |
| | | $ZnCl_2$ | 500 | 2.2 | ± | 0.1 | 47 | % | + |
| **Root** | Mo | Control | *** | 16.0 | ± | 1.6 | 100 | % | |
| | | Z-COTE HP1 | 500 | 28.1 | ± | 2.0 | 76 | % | + |
| | | $ZnCl_2$ | 500 | 0.8 | ± | 0.5 | 95 | % | - |
| | B | Control | *** | 8.5 | ± | 1.3 | 100 | % | |
| | | Z-COTE | 250 | 18.8 | ± | 0.6 | 121 | % | + |
| | | Z-COTE HP1 | 250 | 18.2 | ± | 1.9 | 114 | % | + |

**Effects in nodules nutrients**

Figure 2.5 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 at 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 (Schultze & Kondorosi, 1998). Nickel has been regarded as an important element in the symbiotic relationship between soybean plant and its rhizobia *(Rhizobium japonicum)* (Klucas *et al.*, 1983). Therefore, the impact of the ZnO could represent a potential risk for the nodules activity, caused by changes in accumulation of nutrients such as Ca and Ni.

Manganese was also reduced by bulk ZnO at 125 mg $kg^{-1}$ by 99.8%, as well as P at 125 mg $kg^{-1}$ 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 (Israel, 1987), and in Medicago truncatula, insufficient P reduced early nodule functioning, along with increased release of protons in roots (Johnson *et al.*, 1996). According to O'Hara (2001), deficiencies in Ca, Fe, and Mo could cause dysfunctional nodule development and activity.

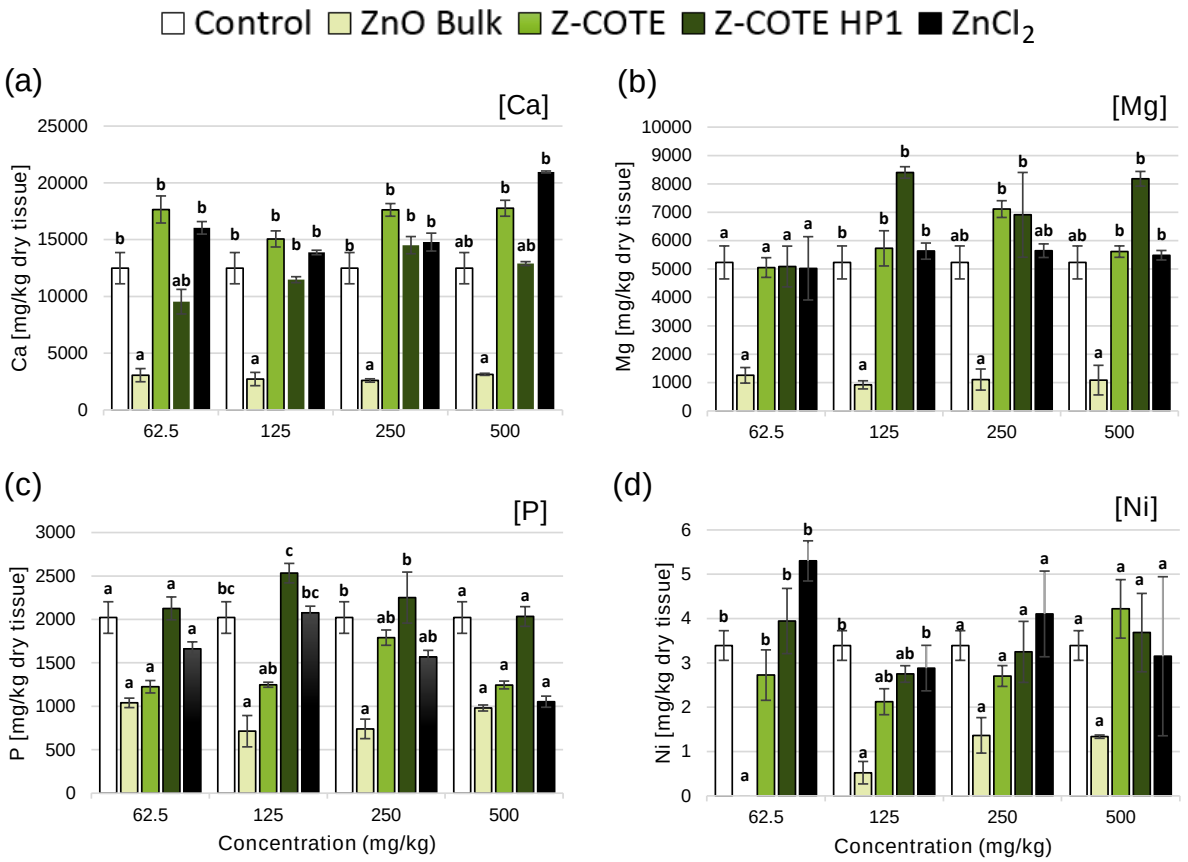


Figure 2.5: Nutrient composition of nodules of red kidney bean plants exposed to Z-COTE, Z-COTE HP1, bulk ZnO, $ZnCl_2$, and DI water (control) at concentrations of 62.5, 125, 250 and 500 mg $kg^{-1}$ of soil. Letters represent statistically significant differences between the mean of the respective control and Zn compounds at the same concentration ($p \leq 0.05$). Bars represent the standard error of treatments (n=16 for controls, n=4 for Zn compounds).

## 2.4 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, $ZnCl_2$ 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 $kg^{-1}$, increased nodule Zn. While bulk ZnO reduced nodule Zn at all concentrations. In leaves, uncoated ZnO at 500 mg $kg^{-1}$ resulted in significantly more Zn, compared with coated ZnO. At 500 mg $kg^{-1}$, bulk ZnO, Z-COTE, and $ZnCl_2$ increased pod Zn by 71%, 60, and 52%, 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). $ZnCl_2$ 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 $ZnCl_2$ imposed more toxicity to kidney beans, since they reduced root and leaf elongation, respectively, and several essential elements in tissues.

# Chapter 3

## Nutritional quality of bean seeds harvested from plants grown in different soils amended with coated and uncoated zinc oxide nanomaterials[2]

## 3.1 Introduction

Although there are many studies about the effects of nanomaterials (NMs) in plants, very few of them have been performed in natural soils. Moreover, responses of plants to NM exposure in soils with different physicochemical properties are limited. Reports indicate that media conditions affect aggregation, dissolution, bioavailability, and mobility of NMs (García-Gómez *et al.*, 2017, Louie *et al.*, 2016, Majumdar *et al.*, 2016, Rodrigues *et al.*, 2016). Of particular importance is the soil natural organic matter (NOM). NOM diminishes aggregation of NMs and increases their stability, which lead to different toxic effects (Grillo *et al.*, 2015, Louie *et al.*, 2016). In addition, the type, concentration, size, and surface functionality of the NMs modulate their mobility in different environments (Josko & Oleszczuk, 2013, Louie *et al.*, 2016).

Zinc oxide (ZnO) manufactured NMs are increasingly being incorporated into consumer goods, especially in personal care products, due to their UV-blocking properties. Commercially available Z-COTE (amphiphilic, uncoated ZnO NM) and Z-COTE HP1 (hydrophobic ZnO NM, coated with triethoxycaprylylsilane) are currently used in a variety of products. The estimated environmental release of ZnO NMs in the U.S. ranges from 1800–2100 me-

[2]Reprinted from Medina-Velo, I. A., Dominguez, O. E., Ochoa, L., Barrios, A. C., Hernandez-Viezcas, J. A., White, J. C., Peralta-Videa, J. R., and Gardea-Torresdey, J. L. (2017) Nutritional quality of bean seeds harvested from plants grown in different soils amended with coated and uncoated zinc oxide nanomaterials. *Environmental Science: Nano* 4(12), 2336-2347. 

tric tons per year, 24-36% of which ends up in soils (Keller *et al.*, 2014). In addition, ZnO NMs are proposed to be used as fertilizers, due to their great reactivity (Zn availability), in comparison to the bulk particles (Milani *et al.*, 2015). This could put an excess of Zn in ecosystems, with unknown consequences for the soil biota (Holden *et al.*, 2016).

Zinc uptake and accumulation in edible portions of plants exposed to ZnO NMs have been reported in wheat (Du *et al.*, 2011), soybean (Hernandez-Viezcas *et al.*, 2013, Peralta-Videa *et al.*, 2014), peanut (Prasad *et al.*, 2012), kidney bean (Medina-Velo *et al.*, 2017a), and cucumber (Zhao *et al.*, 2013a). A short-term comparative study of ZnO NMs, bulk ZnO, and $ZnSO_4$ in common bean *(Phaseolus vulgaris)* plants grown in acidic soil (pH 5.4) and calcareous soil (pH 8.3) showed that effects in antioxidant defenses were dependent on the exposure time and soil type, while the Zn availability did not differ among the Zn materials (García-Gómez *et al.*, 2017).

However, to the best of the authors' knowledge, there are no reports about the effect of coated ZnO NMs in soil cultivated common beans. Moreover, none of the available reports describe the effects of exposure of such NMs on soils with different organic matter (OM) contents along a full-life cycle. The working hypothesis is that in natural soil (NS), Zn availability for plants exposed to Z-COTE HP1 would be lower than those exposed to Z-COTE, due to the coating's hydrophobicity, while in organic matter-enriched soil (ES), there will be no difference in Zn availability, due to the presence of OM.

Hence, the objectives of this study were to compare the effects of surface coating and the particle size of ZnO in the nutritional composition of common beans. The plants were exposed to bare and surface coated nanosized ZnO, microsized (bulk) ZnO, and ionic $ZnCl_2$ for the full life cycle. At harvest, the seeds were analyzed using different techniques to determine the treatment effects in the nutritional composition of the bean seeds.

## 3.2 Experimental section

### 3.2.1 Nanomaterials and compounds

ZnO NMs (Z-COTE and Z-COTE HP1) were purchased from BASF, bulk ZnO ACS reagent ≥99.0% purity from Sigma-Aldrich, and $ZnCl_2$ ACS reagent ≥97% purity from Acros Organics. Nanomaterial characterization was included in a previous report (Medina-Velo *et al.*, 2017a). Red hawk kidney bean *(Phaseolus vulgaris)* seeds were provided by Dr. James Kelly from Michigan State University and stored at 4°C until use.

### 3.2.2 Soil characterization

In this study, we used medium loam (19% clay, 44% silt, 36% sand) NS, collected from a cotton agricultural field in Socorro, TX, USA (N 31° 40.489, W 106° 17.198, elevation: 1115 MASL) and the Miracle Grow potting mix, purchased from a local store. The original materials were sieved through an 8 mm mesh to dispose of large clots, rocks, and wood chips. The ES was a mixture of 50% NS with 50% potting mix (w/w). Both the NS and ES were characterized for OM, pH, total dissolved solids (TDS), electrical conductivity (EC), and microelement composition.

The organic matter content was determined in four replicates of each soil type by comparing the weight of the soil before and after ignition at 375°C for 16 h (loss-on-ignition method) (Ball, 1964).

Measurements of pH, TDS, and EC were conducted using a portable pH/EC/TDS/temperature meter (HI 9811-5 Hanna instruments, Woonsocket, RI, USA). Three replicates of 10 g (1:2, soil:water) of NS or 5 g (1:4, soil:water) of ES were suspended in 20 mL of deionized water (DW) or 0.01 M $CaCl_2$ (Schofield & Taylor, 1955). Samples were manually mixed with a glass rod for 90 s and allowed to settle for 30 min. After repeating the process three times, readings were taken by introducing the instrument probe for 1 minute to the liquid phase.

For microelement determination, three replicates of 0.15 g of each soil were digested with 4 mL of aqua regia in a DigiPREP block digestion system (SCP Science, Quebec, Canada) at 115°C for 45 min (USEPA method 3050b) and diluted to 50 mL with ultrapure water (UPW, 18.2 MΩ $cm^{-1}$). The digested samples were analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES, PerkinElmer Optima 4300 DV, Shelton, CT, USA). The ICP-OES parameters used were as follows: nebulizer flow, 0.80 L $min^{-1}$; power, 1300 W; peristaltic pump rate, 1.5 mL $min^{-1}$; flush time, 15 s; delay time, 20 s; read time, 10 s; and wash time, 60 s.

### 3.2.3 Soil amendment and bean growth

For soil amendment, each compound was weighed and suspended in 100 mL of DW to achieve concentrations of 62.5, 125, 250 and 500 mg of compound per kg of soil. Z-COTE and bulk ZnO suspensions were water bath sonicated (Crest Ultrasonics, Trenton, NJ, US) for 30 min at 25°C, and an intensity of 180 watts, before being added to the soil. Z-COTE HP1 and $ZnCl_2$ suspensions/solutions were added to the soil without sonication. Four replicates of each concentration and four controls were prepared for each compound, in each soil type. There were 40 treatments with 4 replicates per treatment (160 pots and a total of 800 plants).

The suspensions were mixed with 1.3 kg of NS or 500 g of ES (amounts varied due to differences in density) using a hand-held cultivator. The amended soil was transferred into plastic pots (12.5 cm diameter × 14 cm height) and allowed to equilibrate for 24 h at room temperature. Red kidney bean *(P. vulgaris)* seeds were disinfected with 2% NaClO and hydrated in DW for 24 h to facilitate germination (Majumdar *et al.*, 2016, Medina-Velo *et al.*, 2017a). Five seeds were planted in a quincunx pattern at 2.5 cm depth, watered with 50 mL of DW, and placed in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH, USA) with 14 h photoperiod (340 $\mu$mole $m^{-2}$ $s^{-1}$), 25/20 °C day/night temperature and 65-70% relative humidity. The pots were watered daily with 50 mL of DW until germination, then, with 100 mL of DW until maturity.

After 45 days of planting, four samples of soil were collected from each replicate. Zinc was quantified using ICP-OES following the methodology previously described.

Once all pods with seeds were mature (amber colored and ≈15% humidity), they were cut from the calyx including the pedicel. The number and weight of pods and seeds were recorded. Seeds and carpels were placed into envelopes and oven dried at 70°C for 72 h.

### 3.2.4 Protein and total sugar analysis

The protein content was analyzed by nitrogen combustion, as per Chang (2014) using a Leco FP628 Nitrogen Determinator (Saint Joseph, MI, USA). Samples were read twice; additionally, LECO reference materials of wheat flour and EDTA were used for quality control and quality assurance (QA/QC). Total sugar extractions were performed as per Dubois *et al.* (1956) and quantified in a microplate colorimetric format of the phenolsulfuric acid method (Masuko *et al.*, 2005).

### 3.2.5 Mineral composition

Accumulation of Zn, K, S, Mg, Ca, P, Fe, Cu, Mn, Mo, and Ni in seeds was quantified. Samples of 0.15 g of powdered seeds were acid-digested with 4 mL of trace pure HNO3 in a DigiPREP block digestion system (SCP Science, Quebec, Canada) at 115°C for 45 min and diluted to 50 mL with UPW. The total metal contents in the samples were then determined using ICP-OES. Standard reference material 1570a from the National Institute of Standards and Technology was used to validate the digestion and analytical method, obtaining a recovery of 101% for Zn. For QC of the ICP-OES readings, multielemental standard solutions of 1 and 20 mg $L^{-1}$ were analyzed every 10 samples, with recoveries of 100 ± 10%.

### 3.2.6 Experimental design and statistical analysis

The experiment was designed with a factorial array, following a random design. The tested factors were soil, at two levels (NS and ES); compound, at 4 levels (bulk ZnO, Z-COTE, Z-COTE HP1, $ZnCl_2$); and concentration at 5 levels (0, 62.5, 125, 250, 500 mg $kg^{-1}$). Data was analyzed using the Statistical Package for the Social Sciences 22 (SPSS, Chicago, IL, USA).

Three-way ANOVA was performed to determine the effects of soil, compounds and concentration on seed production and nutrient composition of seeds. Statistical significance was accepted at a p-value of 0.05. Simple-simple pairwise comparisons were run with a Bonferroni adjustment to identify differences in individual treatments by a single main effect (soil, compound or concentration). Data are mean ± standard deviation (SD) or standard error (SE) as stated in tables/figures of specified number of replicates. In addition, relative values were calculated for protein and sugar (equation 3.1), and the relative increase/decrease was used for further statistical analysis.

Relative value (%) = (actual value / control value) × 100 (equation 3.1)

## 3.3 Results and discussion

### 3.3.1 Nanomaterial characterization

Z-COTE and Z-COTE HP1 NMs were previously characterized in suspension forms (Medina-Velo *et al.*, 2017a). The primary size, phase and structure, shape, surface area, zeta potential, and purity are summarized in Table 2.1.

Differences in the specific surface area, size, and zeta potential of Z-COTE and Z-COTE HP1 may modify their availability to interact with soil components. For instance, the larger surface area of Z-COTE contains additional absorption sites that potentially increase the binding capacity to the soil components (Rodrigues *et al.*, 2016), in comparison to the coated Z-COTE HP1. On the other hand, the negative surface charge of Z-COTE HP1

increases its stability, but changes the way it interacts with hydrophobic and hydrophilic surfaces in the soil and plant systems. The small size of Z-COTE endows it with better solubility rates than its bulk counterpart (Rodrigues *et al.*, 2016).

### 3.3.2 Soil characteristics

Table 3.1 summarizes the physicochemical characteristics of NS and ES. The enrichment of NS with the commercial potting mix increased the OM from 2.8% (NS) to 18.0% (ES). Organic matter enrichment not only improves nutrient availability, but it also creates different conditions that modify the way ZnO NMs interact with the surroundings. In addition, functional groups in the OM provide binding sites for the formation of complexes with the Zn compounds, increasing their bioavailability (Zeng *et al.*, 2011).

The soil pH decreased from 7.8 to 6.8 point after OM addition. This change in pH represented a transition from alkaline to close to neutral (Table 3.1), which could possibly lead to aggregation, dissolution or changes in the adsorption of OM components into the surface of the NMs. The transition to a lower pH also represents an increase in Zn and other metals' mobility (Rusjan, 2012). A pH between 6.5 and 7.0 is optimum for legume production (van Schoonhoven & Voysest, 1991, Verheye, 2010). Thus, the ES represented a better environment for plant growth. A change in soil pH also modifies the interaction of the hydrophobic and hydrophilic fractions of the OM with the NMs (Grillo *et al.*, 2015).

As expected, TDS were higher in the ES, with an increase of 94%, when measured in $H_2O$, and 32% for $CaCl_2$ determination, compared with the NS.

The mineral composition was also different in both soil types. The most significant change was found in the P content that increased by 70% in the ES, compared with that in the NS, while Zn was 13% lower in the ES than that in the NS. Higher amounts of the soil P content could reduce the phytoavailability of Zn ions,6 which may suggest less uptake and translocation of Zn in the ES, compared to that in the NS. The amounts of Al and Fe were diminished after OM enrichment (51% and 24%, respectively), whose oxides represent potential absorption sites for NOM (Zhao *et al.*, 2012), enhancing the availability

of organic compounds in the ES.

In addition, the Zn content in the soil samples after 45 days of growth showed consistently higher levels than that in the controls (Fig. 3.1).

Table 3.1: Characterization summary of natural soil (NS) and organic matter-enriched soil (ES)

| **Characteristic** | | **Natural soil (NS)** | **Enriched soil (ES)** |
|---|---|---|---|
| Organic matter (OM) [%] | | 2.8 ± 0.2 | 18.0 ± 0.8 |
| pH | $H_2O$ | 7.8 ± 0.06 | 6.8 ± 0.0 |
| | $CaCl_2$ | 7.7 ± 0.0 | 6.7 ± 0.0 |
| Total dissolved solids (TDS) [mg $L^{-1}$] | $H_2O$ | 703 ± 21 | 1370 ± 131 |
| | $CaCl_2$ | 1423 ± 15 | 1877 ± 147 |
| Electrical conductivity (EC) [$\mu$S $cm^{-1}$] | $H_2O$ | 1430 ± 36 | 2750 ± 262 |
| | $CaCl_2$ | 1853 ± 25 | 3760 ± 293 |
| K [mg $kg^{-1}$] | | 35497 ± 178 | 38114 ± 960 |
| S [mg $kg^{-1}$] | | N/D | 44.1 ± 1.8 |
| Ca [mg $kg^{-1}$] | | 29919 ± 135 | 27929 ± 1139 |
| Mg [mg $kg^{-1}$] | | 6416 ± 64 | 5979 ± 53 |
| Mn [mg $kg^{-1}$] | | 432 ± 4 | 423 ± 4 |
| Fe [mg $kg^{-1}$] | | 14964 ± 203 | 12066 ± 234 |
| Zn [mg $kg^{-1}$] | | 62 ± 8 | 54 ± 2 |
| Cu [mg $kg^{-1}$] | | 27 ± 2 | 23 ± 0 |
| P [mg $kg^{-1}$] | | 686 ± 6 | 1170 ± 10 |
| Al [mg $kg^{-1}$] | | 14445 ± 225 | 9584 ± 155 |

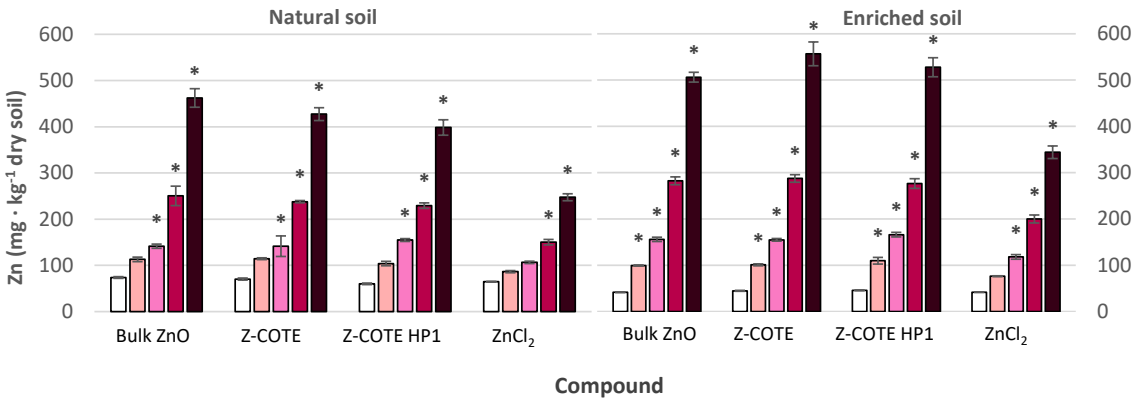


Figure 3.1: Zinc content in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$ after 45 days of growth of red kidney bean plants. Values are average ± standard deviation of 4 replicates. *Represents treatments different from their respective control.

### 3.3.3 Maturation time and yield

#### Number of days to harvest maturity

The number of days from planting to harvesting the mature seeds is shown in Table 3.2. The statistical analysis showed that none of the individual treatments, at any concentration, were different from their controls. However, the effect of the soil type, as a main factor, was observed. In addition, the three-way ANOVA showed a significant interaction of soil × Zn compounds.

The average life cycle of red kidney beans is 99 (95–102) days (www.centralbean.com/seed.html). As seen in Fig.3.2, NS plants took longer (112 ± 8 days) to reach maturity than the ES plants (87 ± 11 days). The longer maturation time recorded in the NS plants is attributed to the reduced OM content in the soil, since it is highly responsible for plant development (Zandonadi *et al.*, 2013). Flower primordia usually start after apexes reach a critical development (Taiz & Zeiger, 2002), a trait that has been accelerated in another crop by the addition of OM (Abu-Zahra, 2012).

Table 3.2: Number of days to harvest maturity for red kidney bean plants seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type, the symbol ∧ represents differences between-soil within the same compound, at the same concentration; *stands for differences against the respective control

| | | | **Natural soil** | | **Enriched soil** |
|---|---|---|---|---|---|
| **Parameter** | **Compound** | **Concentration (mg $kg^{-1}$)** | $\overline{x}$ ± **SD** | | $\overline{x}$ ± **SD** |
| **Days to harvest maturity (#)** | **Bulk ZnO** | Control | 109.8 ± 2.9 | ∧ | 92.0 ± 3.5 |
| | | 62.5 | 108.8 ± 4.5 | | 99.0 ± 4.6 |
| | | 125 | 109.5 ± 6.1 | ∧ | 86.5 ± 14.8 |
| | | 250 | 109.0 ± 4.5 | | 95.0 ± 0.0 |
| | | 500 | 102.8 ± 10.3 | | 91.5 ± 14.2 |
| | **Z-COTE** | Control | 106.8 ± 3.9 | | 94.8 ± 10.9 |
| | | 62.5 | 105.5 ± 4.1 | ∧ | 88.3 ± 13.7 |
| | | 125 | 105.0 ± 8.0 | ∧ | 82.8 ± 15.9 |
| | | 250 | 104.5 ± 1.7 | | 92.3 ± 5.1 |
| | | 500 | 101.8 ± 10.1 | ∧ | 81.3 ± 11.3 |
| | **Z-COTE HP1** | Control | 122.3 ± 13.6 | ∧ | 83.0 ± 8.0 |
| | | 62.5 | 110.5 ± 16.6 | ∧ | 83.3 ± 15.9 |
| | | 125 | 116.8 ± 9.5 | ∧ | 82.0 ± 17.1 |
| | | 250 | 121.8 ± 4.5 | ∧ | 81.8 ± 11.0 |
| | | 500 | 115.5 ± 17.0 | ∧ | 84.0 ± 18.6 |
| | **$ZnCl_2$** | Control | 123.5 ± 5.7 | ∧ | 87.5 ± 11.6 |
| | | 62.5 | 124.5 ± 9.1 | ∧ | 85.3 ± 11.3 |
| | | 125 | 117.0 ± 12.8 | ∧ | 86.8 ± 10.2 |
| | | 250 | 123.8 ± 9.0 | ∧ | 87.5 ± 7.1 |
| | | 500 | 111.0 ± 0.0 | ∧ | 81.3 ± 9.0 |

NS plants exposed to Z-COTE HP1 and $ZnCl_2$ took ≈12 days more than the plants grown in soil amended with bulk ZnO and Z-COTE. In *Arabidopsis thaliana*, flowering was retarded by presence of choline chloride (Chandler & Dean, 1994), which could have been possibly formed from the Cl released by $ZnCl_2$, retarding the flowering in NS bean plants. Conversely, ES plants exposed to Z-COTE HP1 required 9% less time (7 days) than those exposed to bulk ZnO.

No effect was found after exposure to Z-COTE, in agreement with a study performed in tomato *(Solanum lycopersicum)*, where plants exposed to ZnO NM at 10-1000 mg $kg^{-1}$ recorded flower and fruit production at day 40, similarly to control plants (Raliya *et al.*, 2015). In our previous study with kidney beans, we observed promotion of plant growth after exposure to the same concentrations of Z-COTE HP1 in NS (Medina-Velo *et al.*, 2017a). This validates the reduction in the time for harvest maturity found in ES, but it is opposite to what happened in NS. It is possible that the Si present in the coating of Z-COTE HP1 particles promoted plant growth in the presence of high OM. Silicon has shown to produce several beneficial effects in plants, including an increase in the flower size and flower product in *Hosta tratt* (Denisow *et al.*, 2015). In addition, Si in OM enriched soil (peat) was referred to improve plant development (Wroblewska & Dkebicz, 2011).

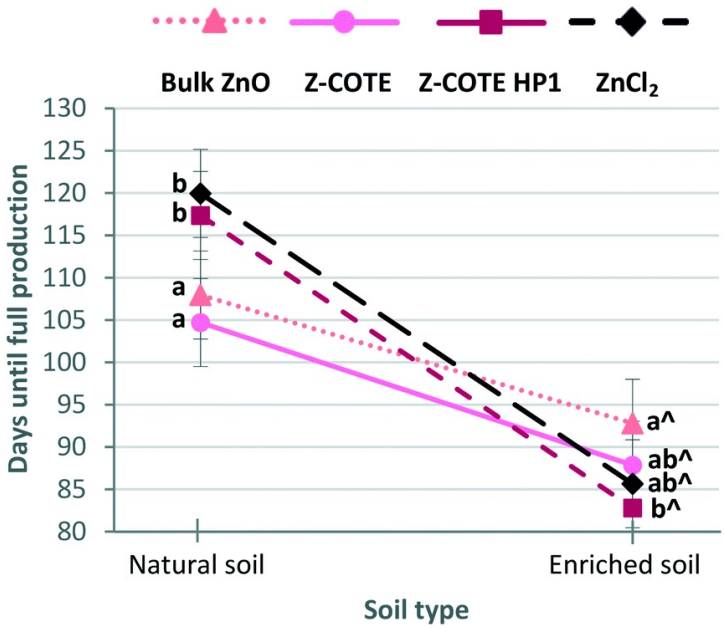


Figure 3.2: Soil × compound interaction plot of number of days to maturity harvest of red kidney beans seeds grown in natural soil (NS) or organic matter-enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1 and $ZnCl_2$. Data are average of 20 replicates (concentration effect is not considered) and SE = 5.221. Letters represent differences between-compounds within the same soil type and the symbol ∧ represents differences between-soil type within the same compound.

### Seed production

Table 3.3 shows the number and fresh weight of seeded pods and seeds. The statistical analysis showed the significant effect of the tested compounds and the soil type.

Pairwise comparisons showed that $ZnCl_2$ in the NS affected the production and biomass of seed-containing pods. Under $ZnCl_2$ exposure, the number of pods with seeds was 44% lower at 500 mg $kg^{-1}$, whereas the biomass of seed-containing pods was reduced at 125 (43%), 250 (48%), and 500 mg $kg^{-1}$ (66%), compared with the control. In our previous

study with kidney beans, $ZnCl_2$ also showed signs of toxicity in terms of plant elongation and relative chlorophyll (Medina-Velo *et al.*, 2017a). Toxicity was mainly attributed to the presence of $Cl^-$, which in a different experiment reduced red kidney bean yield to 39% after exposure to 80 meq $L^{-1}$(233.86 mg $L^{-1}$) of NaCl (Hajrasuliha, 1980). No significant changes were found between individual treatments in ES and their respective controls. With the exception of $ZnCl_2$, Zn addition to the alkaline ES did not have an impact on seed production, in concordance with a study that reported no significant yield decrease in field bean *(P. vulgaris)* grown in alkaline soil treated with up to 500 mg $kg^{-1}$ of bulk Zn (Boawn & Rasmussen, 1971). However, there was a concentration-dependent slight decrease in the number and weight of seeds for the plants exposed to ES or NS + $ZnCl_2$. This suggests that the response could be different in soil with higher OM content.

Regarding the soil effects, most of the production values were significantly higher in the ES than those in the NS. The number of pods with seeds was 42% higher in the ES than that in the NS, and the weight of seed-containing pods was ≈134% higher in the ES than that in the NS. In addition, those in the ES produced ≈101% more seeds, with a ≈155% increase in seeds' fresh weight, compared to those in the NS. The increase in the yield was attributed to the soil OM enrichment, which is important for plant development (Pinto *et al.*, 2004, Zandonadi *et al.*, 2013). The ES also had higher P availability that has been associated with higher yield in common bean cultivars (Araújo & Teixeira, 2008).

The effect of the tested compounds showed that plants grown in the NS with 500 mg $kg^{-1}$ of $ZnCl_2$ produced 53% less pods with seeds, compared with the plants exposed to Z-COTE at the same concentration, and 45% lighter pods than the plants from the bulk ZnO treatment.

Moreover, NS + $ZnCl_2$ at 500 mg $kg^{-1}$ reduced the number of seeds by 46%, compared with bulk ZnO at the same concentration. On the other hand, ES + $ZnCl_2$ at 250 mg $kg^{-1}$ produced 74% and 75% lighter seeds, compared to bulk ZnO and Z-COTE HP1, respectively. Perhaps the formation of choline chloride not only retarded the flowering but also interfered with the accumulation of other nutrients.

Table 3.3: Seeds and pods with seeds in red kidney bean plants grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control

| | | | Natural soil | | Enriched soil | | Natural soil | | Enriched soil |
|---|---|---|---|---|---|---|---|---|---|
| **Parameter** | **Compound** | **Concentration (mg $kg^{-1}$)** | $\overline{x}$ ± **SD** | | $\overline{x}$ ± **SD** | | $\overline{x}$ ± **SD** | | $\overline{x}$ ± **SD** |
| **Number of pods with seeds (#)** | **Bulk ZnO** | **Control** | 5.0 ± 0.8 | ∧ | 8.5 ± 1.7 | **Weight of pods with seeds (g)** | 3.605 ± 0.433 | ∧ | 8.730 ± 1.064 |
| | | **62.5** | 5.3 ± 1.0 | ∧ | 9.3 ± 1.5 | | 3.014 ± 0.286 | ∧ | 9.243 ± 0.889 |
| | | **125** | 5.0 ± 0.8 | ∧ | 8.0 ± 0.8 | | 3.648 ± 1.008 | ∧ | 8.521 ± 1.501 |
| | | **250** | 5.3 ± 1.5 | ∧ | 8.3 ± 1.3 | | 3.263 ± 1.036 | ∧ | 9.579 ± 0.915 |
| | | **500** | 5.5 ± 0.6 ab | | 7.3 ± 1.7 | | 3.943 ± 0.640 a | ∧ | 8.465 ± 0.789 |
| | **Z-COTE** | **Control** | 6.3 ± 1.7 | ∧ | 9.3 ± 1.3 | | 4.004 ± 1.186 | ∧ | 8.612 ± 0.452 |
| | | **62.5** | 5.8 ± 1.9 | ∧ | 8.3 ± 0.5 | | 4.286 ± 1.794 | ∧ | 8.894 ± 0.687 |
| | | **125** | 7.0 ± 0.8 | | 7.0 ± 2.2 | | 3.941 ± 0.370 | ∧ | 8.340 ± 1.435 |
| | | **250** | 5.8 ± 0.5 | ∧ | 8.8 ± 1.3 | | 3.929 ± 0.773 | ∧ | 9.320 ± 1.679 |
| | | **500** | 6.5 ± 1.3 a | | 8.3 ± 1.0 | | 3.553 ± 1.045 ab | ∧ | 8.622 ± 0.381 |
| | **Z-COTE HP1** | **Control** | 5.8 ± 1.0 | ∧ | 7.8 ± 1.0 | | 4.182 ± 0.478 | ∧ | 8.975 ± 0.729 |
| | | **62.5** | 5.8 ± 0.5 | | 6.8 ± 1.0 | | 4.531 ± 0.424 | ∧ | 9.527 ± 0.476 |
| | | **125** | 5.5 ± 1.7 | | 7.0 ± 1.4 | | 3.790 ± 1.016 | ∧ | 8.922 ± 0.749 |
| | | **250** | 5.0 ± 0.8 | | 6.5 ± 0.6 | | 3.956 ± 0.231 | ∧ | 8.724 ± 1.087 |
| | | **500** | 5.3 ± 1.7 ab | ∧ | 7.3 ± 0.5 | | 3.354 ± 1.140 ab | ∧ | 8.505 ± 1.367 |
| | **$ZnCl_2$** | **Control** | 6.3 ± 1.3 | | 6.8 ± 1.3 | | 5.277 ± 1.561 | ∧ | 8.158 ± 0.641 |
| | | **62.5** | 5.3 ± 2.5 | ∧ | 8.5 ± 1.3 | | 3.313 ± 2.076 | ∧ | 8.572 ± 1.219 |
| | | **125** | 4.8 ± 1.9 | ∧ | 8.8 ± 1.7 | | *3.014 ± 1.800 | ∧ | 7.646 ± 0.625 |
| | | **250** | 4.8 ± 1.0 | ∧ | 6.8 ± 1.0 | | *2.724 ± 0.711 | ∧ | 7.697 ± 0.422 |
| | | **500** | *3.5 ± 1.7 b | ∧ | 7.5 ± 1.3 | | *1.816 ± 1.253 b | ∧ | 8.096 ± 0.583 |
| **Number of seeds (#)** | **Bulk ZnO** | **Control** | 6.0 ± 0.8 | ∧ | 13.8 ± 2.1 | **Weight of seeds (g)** | 2.219 ± 0.262 | ∧ | 5.729 ± 0.641 |
| | | **62.5** | 5.5 ± 1.9 | ∧ | 14.3 ± 2.9 | | 1.876 ± 0.173 | ∧ | 5.598 ± 1.097 |
| | | **125** | 7.5 ± 1.9 | ∧ | 13.3 ± 3.4 | | 2.289 ± 0.641 | ∧ | 5.443 ± 1.190 |
| | | **250** | 5.5 ± 1.3 | ∧ | 15.0 ± 1.6 | | 1.975 ± 0.615 | ∧ | 6.162 ± 0.369 a |
| | | **500** | 7.5 ± 1.0 a | ∧ | 12.0 ± 0.8 | | 2.400 ± 0.393 | ∧ | 5.347 ± 0.826 |
| | **Z-COTE** | **Control** | 7.8 ± 2.1 | ∧ | 13.5 ± 2.6 | | 2.284 ± 0.688 | ∧ | 5.136 ± 0.408 |
| | | **62.5** | 7.5 ± 3.1 | ∧ | 14.0 ± 3.2 | | 2.605 ± 1.244 | ∧ | 5.848 ± 0.566 |
| | | **125** | 7.8 ± 1.7 | ∧ | 12.8 ± 3.8 | | 2.359 ± 0.203 | ∧ | 5.371 ± 1.446 |
| | | **250** | 7.0 ± 0.8 | ∧ | 15.3 ± 3.6 | | 2.174 ± 0.428 | ∧ | 6.034 ± 1.309 ab |
| | | **500** | 7.3 ± 1.3 ab | ∧ | 13.8 ± 1.5 | | 2.361 ± 0.698 | ∧ | 5.594 ± 0.246 |
| | **Z-COTE HP1** | **Control** | 7.3 ± 1.7 | ∧ | 13.3 ± 0.5 | | 2.516 ± 0.473 | ∧ | 6.058 ± 0.662 |
| | | **62.5** | 9.0 ± 0.8 | ∧ | 14.3 ± 1.5 | | 2.804 ± 0.437 | ∧ | 6.508 ± 0.410 |
| | | **125** | 7.0 ± 2.2 | ∧ | 13.3 ± 2.1 | | 2.140 ± 0.577 | ∧ | 6.011 ± 0.601 |
| | | **250** | 6.5 ± 2.4 | ∧ | 14.0 ± 2.7 | | 2.378 ± 0.182 | ∧ | 6.141 ± 0.949 a |
| | | **500** | 6.8 ± 1.0 ab | ∧ | 12.8 ± 2.1 | | 1.962 ± 0.602 | ∧ | 5.713 ± 1.017 |
| | **$ZnCl_2$** | **Control** | 8.3 ± 1.7 | ∧ | 11.8 ± 1.0 | | 2.974 ± 0.911 | ∧ | 5.257 ± 0.350 |
| | | **62.5** | 6.3 ± 3.1 | ∧ | 14.0 ± 2.2 | | 2.072 ± 1.329 | ∧ | 5.814 ± 1.454 |
| | | **125** | 5.5 ± 2.6 | ∧ | 12.5 ± 1.7 | | 1.839 ± 1.171 | ∧ | 4.662 ± 0.231 |
| | | **250** | 5.5 ± 1.3 | ∧ | 12.3 ± 0.5 | | 1.735 ± 0.404 | ∧ | 4.651 ± 0.543 b |
| | | **500** | 3.5 ± 1.7 b | ∧ | 14.5 ± 1.9 | | 1.109 ± 0.840 | ∧ | 5.412 ± 0.536 |

### 3.3.4 Protein and sugar contents

Protein and total sugar contents were quantified in mature seeds, and the data are presented as relative sugar/protein content, which were normalized to the sugar/protein content in control seeds.

#### Protein content

The results for the protein content, the most valuable nutrient in bean seeds, are shown in Table 3.4. The relative protein content is summarized in Fig. 3.3 The statistical analysis of the relative protein content showed the significant effect of Zn compounds and concentration, and the interactions soil × concentration and compound × concentration, in addition to the triple interaction of the evaluated factors.

As observed in Fig. 3.3 the only individual treatment that differed from the control was ES + $ZnCl_2$ at 125 mg $kg^{-1}$, which showed a reduction of 10% in the relative protein content. Pairwise comparisons showed a significant decrease in the relative protein content in seeds grown in NS amended with $ZnCl_2$ at the two highest concentrations, in comparison with bulk ZnO and Z-COTE HP1, whereas, the same reductions in ES were only observed in comparison to bulk ZnO. A study in green beans *(P. vulgaris)* exposed to salt stress conditions (NaCl) showed a decrease in the protein content, with a substantial difference when grown in different sources of nitrogen Pessarakli *et al.* (1989), similarly to 23 cultivars of asparagus bean (*Vigna unguiculata* L. spp. sesquipedalis Verdc) (Chen *et al.*, 2007). Even when $ZnCl_2$ impacted the protein accumulation in both soils, it is important to point out that the average protein content found in this study resulted slightly higher (≈9%) than the standard reference for red kidney bean seeds (22.53 ± 0.225 g 100 $g^{-1}$ dry seeds) (USDA, 2015).

Table 3.4: Protein content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control

| | | | **Natural soil** | | | **Enriched soil** | | |
|---|---|---|---|---|---|---|---|---|
| **Nutrient** | **Compound** | **Concentration (mg $kg^{-1}$)** | $\overline{x}$ ± **SD** | | | $\overline{x}$ ± **SD** | | |
| **Protein** | **Bulk ZnO** | **Control** | 23.689 ± 1.752 | a | | 23.419 ± 1.921 | | |
| **(g 100$g^{-1}$)** | | **62.5** | 22.621 ± 0.779 | a | ∧ | 24.518 ± 1.287 | | |
| | | **125** | 25.869 ± 2.168 | | ∧ | 23.530 ± 2.315 | | |
| | | **250** | 25.146 ± 1.983 | ab | | 25.611 ± 2.035 | a | |
| | | **500** | 24.926 ± 1.966 | | ∧ | 22.623 ± 1.495 | | |
| | **Z-COTE** | **Control** | 26.235 ± 0.906 | b | ∧ | 23.919 ± 2.745 | | |
| | | **62.5** | 25.678 ± 1.642 | b | | 24.494 ± 0.226 | | |
| | | **125** | 25.868 ± 2.281 | | ∧ | 22.944 ± 0.959 | | |
| | | **250** | 25.564 ± 1.603 | ab | | 23.848 ± 0.856 | ab | |
| | | **500** | 23.981 ± 2.442 | | | 22.431 ± 0.698 | | |
| | **Z-COTE HP1** | **Control** | 24.834 ± 2.578 | ab | | 23.641 ± 1.865 | | |
| | | **62.5** | 24.670 ± 0.832 | ab | | 24.736 ± 1.358 | | |
| | | **125** | 24.359 ± 0.574 | | | 24.100 ± 1.783 | | |
| | | **250** | 27.208 ± 1.772 | a | ∧ | 23.143 ± 0.855 | b | |
| | | **500** | 25.778 ± 1.181 | | | 24.306 ± 2.521 | | |
| | **$ZnCl_2$** | **Control** | 26.349 ± 1.181 | b | | 25.526 ± 0.918 | | |
| | | **62.5** | 25.704 ± 2.747 | b | | 24.586 ± 2.111 | | |
| | | **125** | 25.140 ± 1.614 | | ∧ | *22.859 ± 2.518 | | |
| | | **250** | 24.449 ± 1.516 | b | | 24.533 ± 2.547 | ab | |
| | | **500** | 24.090 ± 1.931 | | | 23.360 ± 1.776 | | |

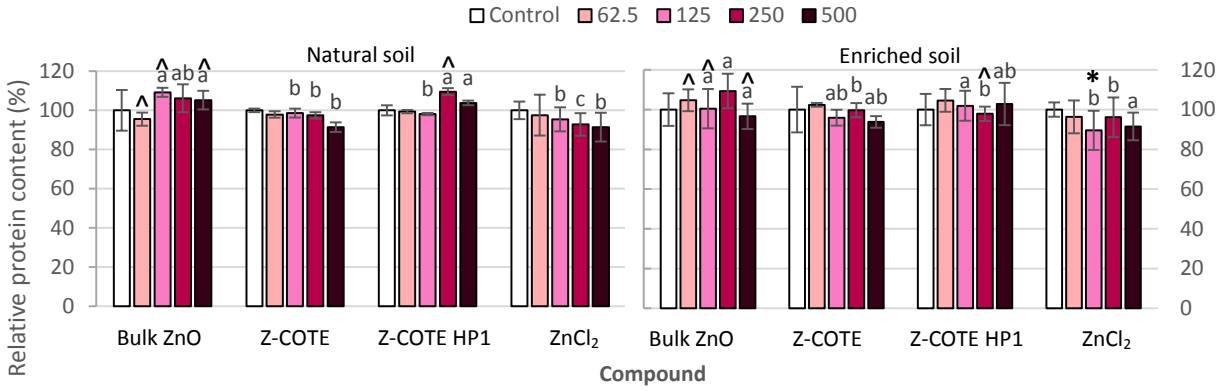


Figure 3.3: Relative protein content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control.

### Sugar content

Zinc is involved in carbohydrate metabolism, including sugar transformation (Sadeghzadeh, 2013). Quantification of total sugar in seeds from the different treatments is shown in Table 3.5. Seeds from all the treatments in this experiment contained higher (up to 182%) amounts of sugar than the average red kidney bean seeds (2.10 g 100 $g^{-1}$ dry seeds)USDA (2015).

As can be observed in Table 3.6, the Zn compounds significantly impacted the relative accumulation of sugar along with the soil × compound interaction. Seeds from ES + $ZnCl_2$ at 62.5, 125 and 250 mg $kg^{-1}$ had higher relative sugar amounts than the controls seeds, with increases of 113%, 148%, and 117%, respectively.

Fig. 3.4 shows that disregarding the concentration, seeds grown in ES amended with

Table 3.5: Sugar content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control

| | | | **Natural soil** | | | | **Enriched soil** | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Nutrient** | **Compound** | **Concentration (mg $kg^{-1}$)** | $\overline{x}$ ± **SD** | | | | $\overline{x}$ ± **SD** | | | |
| **Sugar** | **Bulk ZnO** | **Control** | 3.199 ± 0.382 | a | ∧ | | 5.152 ± 1.236 | a | | |
| **(g $100^{-1}$)** | | **62.5** | 3.025 ± 0.551 | a | ∧ | | 6.338 ± 3.333 | a | | |
| | | **125** | 3.276 ± 0.7 | | | | 4.25 ± 2.661 | | | |
| | | **250** | 3.468 ± 0.359 | a | | | 3.778 ± 0.685 | | | |
| | | **500** | 3.99 ± 1.303 | | | | 3.061 ± 1.318 | | | |
| | **Z-COTE** | **Control** | 6.9 ± 0.926 | b | ∧ | | 3.276 ± 0.515 | ab | | |
| | | **62.5** | 5.876 ± 1.681 | b | ∧ | | 2.91 ± 1.114 | b | | |
| | | **125** | 4.548 ± 1.739 | | ∧ | | 1.884 ± 0.571 | | | |
| | | **250** | 6.683 ± 0.761 | b | ∧ | | 2.574 ± 0.498 | | | |
| | | **500** | 5.624 ± 0.962 | | ∧ | | 1.777 ± 0.476 | | | |
| | **Z-COTE HP1** | **Control** | 3.698 ± 1.302 | a | | | 2.152 ± 0.489 | b | | |
| | | **62.5** | 5.212 ± 2.444 | ab | ∧ | | 2.457 ± 0.466 | b | | |
| | | **125** | 4.594 ± 1.504 | | ∧ | | 2.256 ± 1.296 | | | |
| | | **250** | 5.774 ± 0.937 | ab | ∧ | | 2.073 ± 1.093 | | | |
| | | **500** | 4.927 ± 0.701 | | ∧ | | 2.053 ± 0.982 | | | |
| | **$ZnCl_2$** | **Control** | 5.376 ± 1.281 | ab | ∧ | | 1.213 ± 0.778 | b | | |
| | | **62.5** | 3.758 ± 1.526 | ab | | | 2.581 ± 0.976 | b | | |
| | | **125** | 4.705 ± 2.049 | | | | 3.004 ± 1.508 | | | |
| | | **250** | 4.58 ± 1.875 | ab | ∧ | | 2.635 ± 1.238 | | | |
| | | **500** | 5.045 ± 1.856 | | ∧ | | 2.106 ± 1.045 | | | |

Table 3.6: Relative sugar content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control

| | | | **Natural soil** | | | **Enriched soil** | | |
|---|---|---|---|---|---|---|---|---|
| **Nutrient** | **Compound** | **Concentration (mg $kg^{-1}$)** | $\overline{x}$ ± **SD** | | | $\overline{x}$ ± **SD** | | |
| **Relative** | **Bulk ZnO** | **Control** | 100 ± 11.95 | | | 100 ± 24 | | |
| **sugar** | | **62.5** | 94.55 ± 17.2 | | | 123.01 ± 64.69 | | a |
| **content** | | **125** | 102.4 ± 21.86 | | | 82.5 ± 51.64 | | a |
| **(%)** | | **250** | 108.38 ± 11.23 | | | 73.34 ± 13.29 | | a |
| | | **500** | 124.7 ± 40.71 | | ∧ | 59.41 ± 25.59 | | a |
| | **Z-COTE** | **Control** | 100 ± 13.42 | | | 100 ± 15.74 | | |
| | | **62.5** | 85.16 ± 24.37 | | | 88.86 ± 34 | | a |
| | | **125** | 65.91 ± 25.2 | | | 57.52 ± 17.44 | | a |
| | | **250** | 96.86 ± 11.03 | | | 78.58 ± 15.21 | | a |
| | | **500** | 81.51 ± 13.94 | | | 54.25 ± 14.53 | | a |
| | **Z-COTE HP1** | **Control** | 100 ± 35.19 | | | 100 ± 22.74 | | |
| | | **62.5** | 140.93 ± 66.09 | | | 114.2 ± 21.69 | | a |
| | | **125** | 124.25 ± 40.67 | | | 104.84 ± 60.23 | | a |
| | | **250** | 156.15 ± 25.34 | | | 96.32 ± 50.78 | | a |
| | | **500** | 133.24 ± 18.94 | | | 95.42 ± 45.63 | | ab |
| | **$ZnCl_2$** | **Control** | 100 ± 23.82 | | ∧ | 100 ± 64.16 | | |
| | | **62.5** | 69.9 ± 28.38 | | ∧ | *212.78 ± 80.45 | | b |
| | | **125** | 87.51 ± 38.12 | | ∧ | *247.65 ± 124.3 | | b |
| | | **250** | 85.19 ± 34.88 | | ∧ | *217.27 ± 102.03 | | a |
| | | **500** | 93.85 ± 34.52 | | ∧ | 173.62 ± 86.19 | | b |

$ZnCl_2$ accumulated the highest relative sugar amounts, with values of 103, 114, and 88% higher than bulk ZnO, Z-COTE and Z-COTE HP1, respectively. Accumulation of soluble sugars has been attributed to counteracting osmotic stress, which has been reported in two barley varieties in response to increased NaCl concentration (Khosravinejad *et al.*, 2009). There is the possibility that the higher accumulation of sugar in $ZnCl_2$ seeds was caused by the presence of $Cl^-$, and not necessarily the Zn. In the NS, the highest relative sugar values were present in seeds exposed to Z-COTE HP1, with increases of about 44%, compared with Z-COTE and $ZnCl_2$. Perhaps the negative charge of Z-COTE HP1 acted as a stress factor or a signal that activated the sucrose transport to the root (Lemoine *et al.*, 2013), enhancing its translocation and accumulation in the seeds.

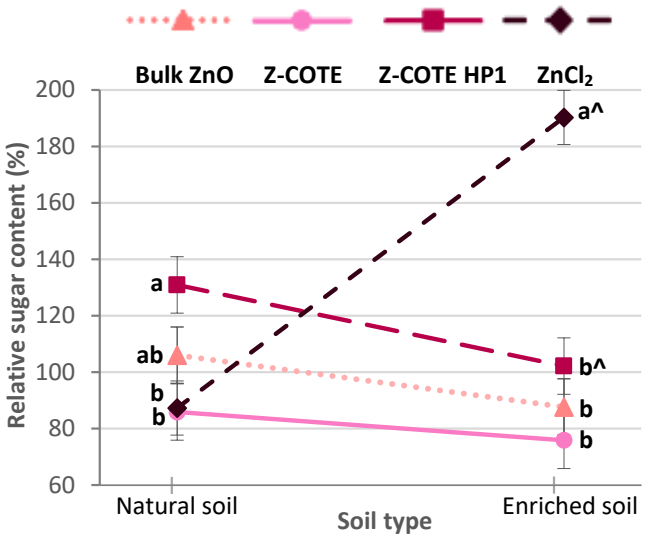


Figure 3.4: Soil × compound interaction plot of relative sugar content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Data are average of 20 replicates (concentration effect is not considered) normalized to the control and SE= 10.092. Letters represent differences between-compounds within the same soil type and the symbol ∧ represents differences between-soil types within the same compound.

### 3.3.5 Essential elements in seeds

#### Zinc

Zinc is classified among the trace minerals in human nutrition, with a demand of about 10 mg per day for adults. Since in this study Zn nanomaterials and compounds were added to the soil, a separate section for Zn uptake is included in Fig. 3.5 and Table 3.7. The three way ANOVA showed significant main effects in Zn accumulation produced by the soil, compound, and concentration, in addition to the interactions of soil × compound and soil × concentration.

Increases of individual treatments with their respective controls showed that seeds from the plants amended with bulk ZnO in the NS had significantly more Zn at 250 (59%) and 500 mg $kg^1$ (47%), compared to the controls, while increases were found in seeds grown in ES + bulk ZnO at 62.5 (55%), 125 (77%), 250 (100%), and 500 mg $kg^1$ (126%). Z-COTE significantly augmented seed Zn at 125, 250 and 500 mg $kg^{-1}$ by 56%, 48%, and 60%, respectively, in the NS, whereas in the ES, the increases were 56%, 81%, 117%, and 143% greater than the controls. The concentration-dependent accumulation of Zn in aerial tissues after exposure to bulk ZnO and/or ZnO NMs has been previously reported in beans (Medina-Velo *et al.*, 2017a), cucumber (Zhao *et al.*, 2014), soybean (Hernandez-Viezcas *et al.*, 2013, Priester *et al.*, 2012), corn (Zhao *et al.*, 2012), and wheat (Du *et al.*, 2011), among others (Zuverza-Mena *et al.*, 2017).

Seeds treated with Z-COTE HP1 showed increases in Zn with respect to the control only at 500 mg $kg^{-1}$ (54%) in the NS, and at all concentrations in the ES, with increases from 66% to 147%. Seeds of plants exposed to $ZnCl_2$ showed higher Zn values than the controls, at 500 mg $kg^{-1}$ in the NS (91%), and at all concentrations in the ES, with increases up to 131%.

Table 3.7: Zinc content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control

| | | | **Natural soil** | | | **Enriched soil** | |
|---|---|---|---|---|---|---|---|
| **Nutrient** | **Compound** | **Concentration (mg $kg^{-1}$)** | $\overline{x}$ ± **SD** | | | $\overline{x}$ ± **SD** | |
| **Zn** | **Bulk ZnO** | **Control** | 48.930 ± 6.879 | | ∧ | 28.765 ± 2.611 | |
| **(mg $kg^{-1}$)** | | **62.5** | ***68.328 ± 5.410** | | ∧ | 44.691 ± 9.360 | |
| | | **125** | 63.483 ± 6.338 | | ∧ | ***51.004 ± 7.536** | ab |
| | | **250** | ***77.846 ± 9.763** | | ∧ | ***57.645 ± 2.799** | |
| | | **500** | ***71.728 ± 8.184** | a | | ***65.098 ± 3.087** | |
| | **Z-COTE** | **Control** | 48.744 ± 1.293 | | ∧ | 25.816 ± 5.060 | |
| | | **62.5** | 62.235 ± 8.220 | | ∧ | 40.174 ± 3.002 | |
| | | **125** | ***75.831 ± 20.037** | | ∧ | ***46.658 ± 10.330** | ab |
| | | **250** | ***72.085 ± 4.112** | | ∧ | ***55.903 ± 8.451** | |
| | | **500** | ***77.773 ± 13.880** | ab | ∧ | ***62.637 ± 3.965** | |
| | **Z-COTE HP1** | **Control** | 54.584 ± 3.627 | | ∧ | 21.613 ± 2.289 | |
| | | **62.5** | 57.707 ± 5.123 | | ∧ | 35.882 ± 4.477 | |
| | | **125** | 64.491 ± 8.837 | | ∧ | 36.873 ± 4.240 | a |
| | | **250** | ***76.136 ± 12.281** | | ∧ | ***46.850 ± 7.426** | |
| | | **500** | ***83.876 ± 9.941** | ab | ∧ | ***53.406 ± 9.600** | |
| | **$ZnCl_2$** | **Control** | 45.636 ± 7.260 | | ∧ | 27.815 ± 3.659 | |
| | | **62.5** | ***65.672 ± 7.761** | | ∧ | ***43.870 ± 5.630** | |
| | | **125** | ***64.845 ± 0.557** | | ∧ | ***53.062 ± 6.885** | b |
| | | **250** | ***62.607 ± 5.261** | | | ***53.907 ± 2.946** | |
| | | **500** | ***87.384 ± 21.696** | b | ∧ | ***64.116 ± 2.867** | |

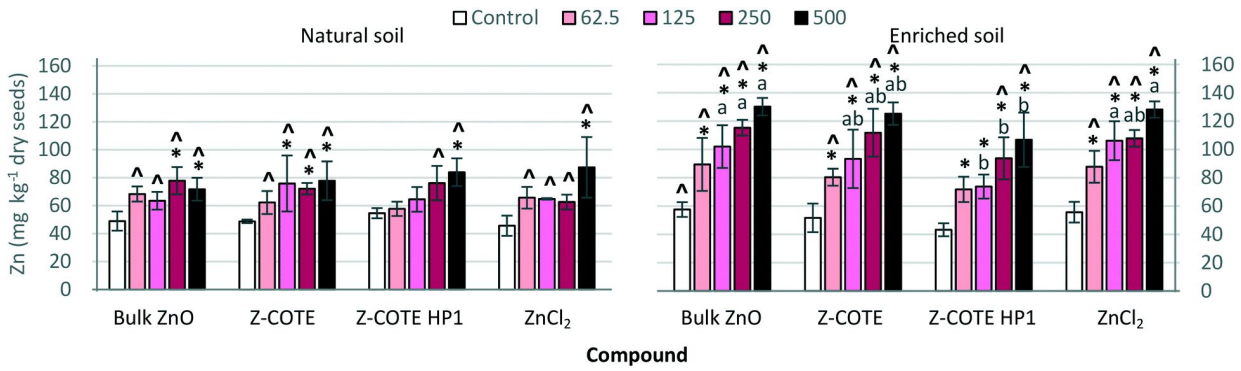


Figure 3.5: Zinc content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control.

Fig. 3.6 shows the significant effect on Zn accumulation in the seeds by the different compounds in the tested soils, independently of the concentration. As can be seen in this figure, seeds from plants grown in the ES accumulated about 38% more Zn than those grown in the NS. The higher Zn seed accumulation in the ES can be attributed to the soil pH, which is lower than the NS pH. A decrease in pH promotes the desorption of soil constituents, such as Zn, Mn, and Fe, which favors their dissolution into the soil solution, enhancing their mobility, and thus, the plant uptake (Zeng *et al.*, 2011). In addition, the organic chemicals present in the higher amount of organic matter of the ES can serve as chelating sites that increase metal availability to plants (Zeng *et al.*, 2011), enhancing uptake and accumulation.

Interestingly, all compounds in the NS accumulated, on average, similar amounts of Zn in seeds (about 67 mg $kg^{-1}$), whereas in the ES, seeds from the Z-COTE HP1 treatment

had about 19% less Zn than the rest of the compounds.

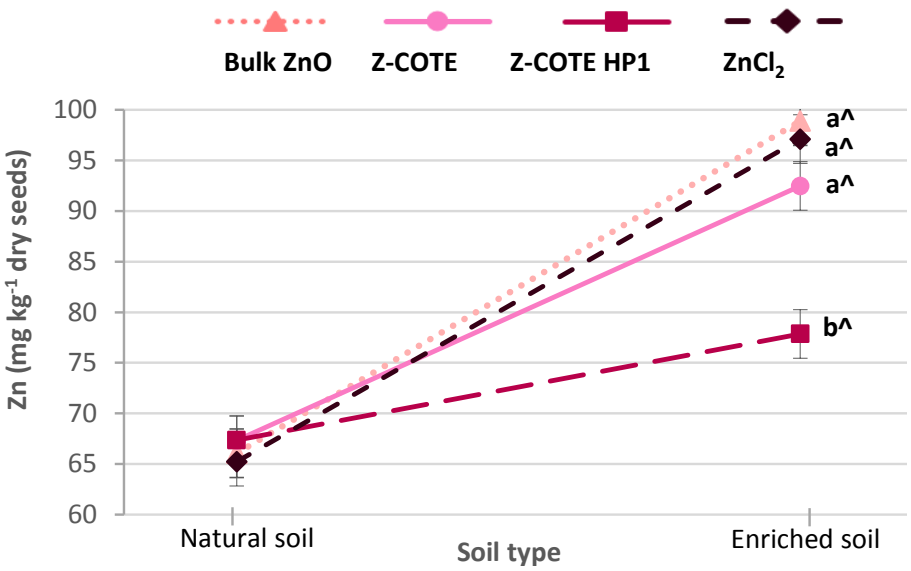


Figure 3.6: Soil × compound interaction plot of Zn content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Data are average ± standard deviation of 20 replicates (concentration effect is not considered); and SE= 2.431. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound.

Finally, the interaction between the soil type and the different concentrations is illustrated in Fig. 3.7 As expected, in both the NS and ES, the seed Zn content increased as the concentration of the tested compounds increased. As has been mentioned, the soil type exerted a significant effect in the accumulation of Zn, since in the ES the values are 30%, 40%, 48%, and 53% higher than those in the NS, at 62.5, 125, 250, and 500 mg $kg^{-1}$, respectively. A positive correlation between the Zn supply and the OM content (Alloway, 2004) was confirmed. However, this effect was not observed in the control seeds, in which Zn accumulation was similar in both soil types.

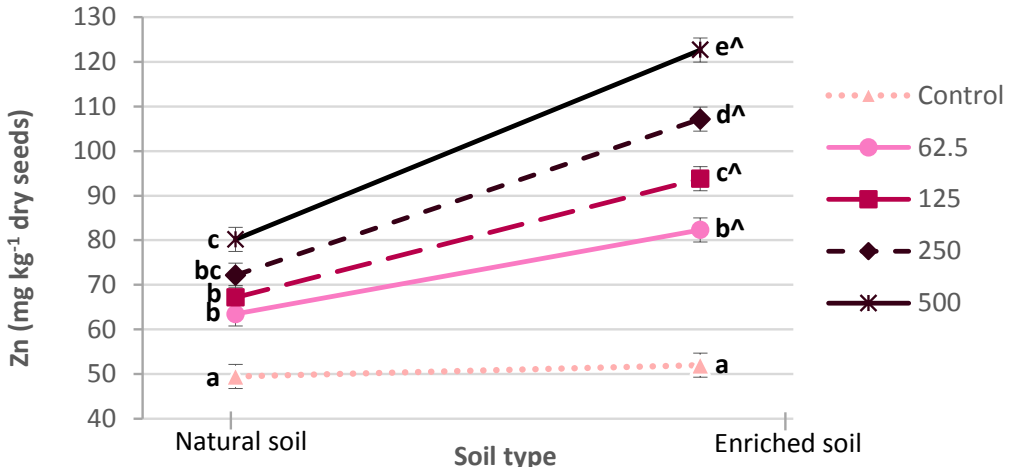


Figure 3.7: Soil × concentration interaction plot of Zn content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Data are average ± standard deviation of 16 replicates (compound effect is not considered); and SE= 2.717. Letters represent differences between-concentrations within the same soil type and the symbol ∧ represents differences between-soil within the same concentration.

### Major minerals

Dietary minerals were quantified in bean seeds, including K, S, P, Mg, and Ca. In general, all major minerals were accumulated in higher amounts in seeds grown in the ES than the seeds grown in the NS. As it was mentioned, the pH (6.8) and OM (18) of the ES represent excellent conditions for the metals to become available to the plants for enhanced uptake, translocation, and accumulation.

Table 3.8 shows the K content in seeds, which was significantly affected by the soil type, Zn compound, and concentration. Moreover, the two-way interaction compound × concentration showed differences in the seed K content. The potassium content was not compromised in any of the treatments, since seeds from this experiment had higher K

values than the average red kidney bean seeds (13590 ± 510 mg $kg^{-1}$) (USDA, 2015) and the seeds grown in the ES had significantly more K (≈64%) than the seeds grown in the NS, attributed to the lower pH and higher OM content, in addition to the presence of more K in the ES.

Comparison of individual treatments with their respective controls showed that $ZnCl_2$ at 500 mg $kg^{-1}$ increased the K content by 25% and 13% in seeds grown in both the NS and ES, respectively. The presence of a low amount of K in soils has been related to a low absorption of Zn in maize and wheat (Biswas *et al.*, 1977); and a study in barley reported increased levels of Zn in the presence of more K and S. The authors proposed a mechanism for Zn absorption mediated by Zn-binding peptides and S-containing proteins (metallothionins), whose production decreases in low S (Pavanasasivam & Axley, 1982). Thus, there is a possible upregulation of metallothionins in the presence of a high amount of Zn in seeds grown under $ZnCl_2$, since the highest seed Zn levels were found after exposure to 500 mg $kg^{-1}$ in both soil types.

Seeds exposed to Z-COTE HP1 showed the lowest K levels in the NS and the ES at 125 and 500, with values about 9% and 10%, respectively, lower than the rest of the compounds. Since the surface charge of Z-COTE HP1 is negative, it is possible that some of the soil K was bound to the Z-COTE HP1, forming a bigger complex and making it less available for plant uptake.

The sulfur concentration in seeds resulted with slight discrepancies between compounds from the same soil; thus, the data were excluded. However, it is important to highlight that as occurred with other major minerals, seeds from the ES treatments showed 44% more S in comparison to those in the NS.

P accumulation in seeds is shown in Table 3.8 and the statistical analysis showed that the main factors, soil and Zn compound, as well as their interaction, significantly affected the P content in the seeds.

The P content in bean seeds grown in NS + $ZnCl_2$ at 500 mg $kg^{-1}$ was 66% higher than that of the controls. A previous study on 45-day old bean plants showed similar results

Table 3.8: Potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca) content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control

| | | | **Concentration (mg kg$^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Nutrient** | **Soil** | **Compound** | **Control** | **62.5 mg kg$^{-1}$** | **125 mg kg$^{-1}$** | **250 mg kg$^{-1}$** | **500 mg kg$^{-1}$** | |
| **K** | **NS** | **Bulk ZnO** | 18473 ± 1635 ∧ | 19455 ± 688 ∧ | 18685 ± 807 ab∧ | 18599 ± 1470 ∧ | 18769 ± 1454 ab∧ | |
| **(mg kg$^{-1}$)** | | **Z-COTE** | 18527 ± 946 ∧ | 17526 ± 905 ∧ | 21552 ± 6191 a∧ | 18037 ± 568 ∧ | 19032 ± 1333 ab∧ | |
| | | **Z-COTE HP1** | 17833 ± 853 ∧ | 17772 ± 572 ∧ | 17883 ± 1137 b∧ | 17213 ± 536 ∧ | 18176 ± 979 b∧ | |
| | | **$ZnCl_2$** | 17538 ± 532 ∧ | 19010 ± 347 ∧ | 18370 ± 2144 ab∧ | 19885 ± 1572 ∧ | ***21872 ± 1932** a∧ | |
| | **ES** | **Bulk ZnO** | 30321 ± 1259 ∧ | 31260 ± 1030 ∧ | 30543 ± 1914 ab∧ | 30451 ± 1230 ∧ | 31531 ± 699 ab∧ | |
| | | **Z-COTE** | 30453 ± 1199 ∧ | 29810 ± 632 ∧ | 31796 ± 2801 a∧ | 30658 ± 2762 ∧ | 31498 ± 1111 ab∧ | |
| | | **Z-COTE HP1** | 29369 ± 1246 ∧ | 29522 ± 1063 ∧ | 27777 ± 1314 b∧ | 30684 ± 3063 ∧ | 29662 ± 1408 b∧ | |
| | | **$ZnCl_2$** | 30530 ± 1089 ∧ | 31102 ± 1419 ∧ | 32339 ± 1738 a∧ | 30771 ± 1621 ∧ | ***34582 ± 901** a∧ | |
| **P** | **NS** | **Bulk ZnO** | 5905 ± 746 ∧ | 7335 ± 998 ∧ | 6382 ± 1751 ∧ | 6505 ± 1947 ab∧ | 6106 ± 1028 a∧ | |
| **(mg kg$^{-1}$)** | | **Z-COTE** | 5778 ± 269 ∧ | 5674 ± 559 ∧ | 6658 ± 1968 ∧ | 5841 ± 485 a∧ | 6293 ± 1543 a∧ | |
| | | **Z-COTE HP1** | 7440 ± 453 ∧ | 6003 ± 795 ∧ | 6969 ± 1627 ∧ | 6140 ± 365 a∧ | 7926 ± 1439 ab∧ | |
| | | **$ZnCl_2$** | 6477 ± 1191 ∧ | 7256 ± 939 ∧ | 6593 ± 1066 ∧ | 9411 ± 1819 b∧ | ***10751 ± 3321** b∧ | |
| | **ES** | **Bulk ZnO** | 13713 ± 386 ∧ | 13497 ± 2317 ab∧ | 13356 ± 2265 b∧ | 13137 ± 475 ∧ | 14086 ± 832 ab∧ | |
| | | **Z-COTE** | 12347 ± 1853 ∧ | 10713 ± 119 a∧ | 11934 ± 3240 ab∧ | 11740 ± 2475 ∧ | 12332 ± 1328 ab∧ | |
| | | **Z-COTE HP1** | 10997 ± 1103 ∧ | 10530 ± 410 a∧ | 10131 ± 1243 a∧ | 11713 ± 2459 ∧ | 11506 ± 2842 a∧ | |
| | | **$ZnCl_2$** | 12959 ± 797 ∧ | 14138 ± 1382 b∧ | 15051 ± 1972 c∧ | 12979 ± 916 ∧ | 14624 ± 1367 b∧ | |
| **Mg** | **NS** | **Bulk ZnO** | 1953 ± 130 ∧ | 2093 ± 115 ∧ | 2022 ± 170 ab∧ | 2060 ± 209 ∧ | 1939 ± 163 ∧ | |
| **(mg kg$^{-1}$)** | | **Z-COTE** | 1996 ± 83 ∧ | 1956 ± 213 ∧ | ***2320 ± 534** b∧ | 1986 ± 51 ∧ | 1952 ± 142 ∧ | |
| | | **Z-COTE HP1** | 1988 ± 162 ∧ | 1964 ± 154 ∧ | 1876 ± 84 a∧ | 2023 ± 128 ∧ | 2159 ± 132 ∧ | |
| | | **$ZnCl_2$** | 1923 ± 41 ∧ | 1904 ± 201 ∧ | 1918 ± 167 a∧ | 1991 ± 111 ∧ | 2162 ± 248 ∧ | |
| | **ES** | **Bulk ZnO** | 3838 ± 48 ∧ | 3851 ± 191 ∧ | 3863 ± 65 ∧ | 3827 ± 52 ∧ | 3955 ± 65 ∧ | |
| | | **Z-COTE** | 3672 ± 230 ∧ | 3564 ± 72 ∧ | 3829 ± 305 ∧ | 3833 ± 303 ∧ | 3674 ± 123 ∧ | |
| | | **Z-COTE HP1** | 3628 ± 184 ∧ | 3623 ± 143 ∧ | 3617 ± 117 ∧ | 3803 ± 289 ∧ | 3722 ± 305 ∧ | |
| | | **$ZnCl_2$** | 3664 ± 164 ∧ | 3732 ± 95 ∧ | 3633 ± 183 ∧ | 3607 ± 171 ∧ | 3810 ± 178 ∧ | |
| **Ca** | **NS** | **Bulk ZnO** | 1066 ± 299 ∧ | 1417 ± 316 ∧ | 1122 ± 345 ∧ | 1410 ± 342 | 952 ± 112 ∧ | |
| **(mg kg$^{-1}$)** | | **Z-COTE** | 1291 ± 247 ∧ | 1185 ± 468 ∧ | 1245 ± 195 ∧ | 1295 ± 76 ∧ | 864 ± 134 ∧ | |
| | | **Z-COTE HP1** | 1149 ± 113 ∧ | 903 ± 74 ∧ | 1042 ± 356 ∧ | 1051 ± 212 ∧ | 1161 ± 388 ∧ | |
| | | **$ZnCl_2$** | 920 ± 131 ∧ | 858 ± 352 ∧ | 1197 ± 201 | 777 ± 297 ∧ | 899 ± 314 | |
| | **ES** | **Bulk ZnO** | 2221 ± 507 ∧ | 2063 ± 707 ∧ | 2061 ± 225 ∧ | 1734 ± 135 | 2384 ± 319 a∧ | |
| | | **Z-COTE** | 2124 ± 294 ∧ | 1770 ± 303 ∧ | 1951 ± 751 ∧ | 1916 ± 562 ∧ | 2003 ± 550 ab∧ | |
| | | **Z-COTE HP1** | 2013 ± 350 ∧ | 1667 ± 304 ∧ | 1773 ± 269 ∧ | 2148 ± 863 ∧ | 2253 ± 786 a∧ | |
| | | **$ZnCl_2$** | 1664 ± 331 ∧ | 1627 ± 85 ∧ | 1653 ± 142 | 1617 ± 332 ∧ | 1378 ± 228 b∧ | |

with increased P in stems and leaves after exposure to 500 mg $kg^{-1}$ of $ZnCl_2$ (Medina-Velo *et al.*, 2017a), attributed to the Zn impact on the expression of the P uptake efficiency (Zhu *et al.*, 2001), possibly caused by the highest levels of seed Zn found in this experiment. Similarly, the soil × compound interaction plotted in Fig. 3.8 shows that seeds grown in NS with $ZnCl_2$, disregarding the concentration, had the highest P accumulation, with values ≈30% higher than bulk ZnO and Z-COTE, whereas in the ES, there was more P in seeds exposed to bulk ZnO (19%) and $ZnCl_2$ (≈23%), in comparison to Z-COTE and Z-COTE HP1. Finally, the P content was 83% more in seeds grown in the ES, than in the NS.

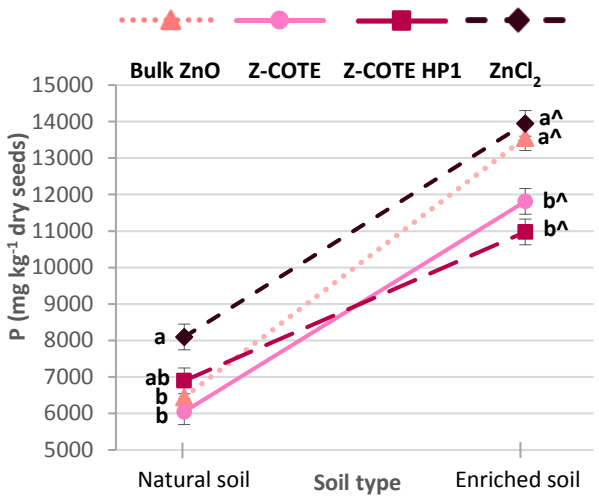


Figure 3.8: Soil × compound interaction plot of P content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Data are average ± standard deviation of 20 replicates (concentration effect is not considered); and SE= 352.485. Letters represent differences between-compounds within the same soil type, and the symbol ∧ represents differences between-soil type within the same compound.

The average Mg content in red kidney bean seeds is 1380 ± 44.5 mg $kg^{-1}$ (USDA, 2015). This suggests that the amounts of Mg found in the treated seeds are higher than

the average, an indicative of healthy seeds. In this study, seeds grown in the ES had ≈86% more Mg than the seeds grown in the NS (Table 3.8). In addition, NS + Z-COTE at 125 mg $kg^{-1}$ significantly augmented the seed Mg content, compared with the control (15%), Z-COTE HP1 (24%), and $ZnCl_2$ (21%). It has been reported that Mg enters the root system through permeable cation channels (White & Broadley, 2009). This suggests that Z-COTE, at 125 mg $kg^{-1}$, activated the $Mg^{2+}$ channels. However, Mg accumulation in seeds was enhanced in the ES, in comparison to that in the NS. More studies are needed in order to clarify these responses.

Similar to Mg accumulation, seed Ca content (Table 3.8) was affected by the soil type and Zn compound. None of the individual treatments resulted statistically different from their respective controls and the Ca amounts were higher than the average Ca content in red kidney bean seeds (830 ± 49.7 mg $kg^{-1}$ (USDA, 2015)).

Calcium levels increased up to 150% in seeds grown in ES + bulk ZnO at concentrations of 0, 62.5, 125, and 500 mg $kg^{-1}$, in comparison to the NS seeds. For $ZnCl_2$, the ES seeds had higher Ca accumulation (up to 108% more) than the NS seeds after exposure to 0, 62.5, and 250 mg $kg^{-1}$. Moreover, for both coated and uncoated Zn NMs, the ES seeds had ≈70% and ≈86% more Ca, respectively, than the NS seeds after exposure to all concentrations. The results suggest a soil effect. The specific response on Ca accumulation caused by the ZnO NMs could be attributed to a size-driven activation of $Ca^{2+}$ channels, which altered the Ca concentration as a response to these stimuli (White & Broadley, 2009). Among the Zn compounds, seeds from $ZnCl_2$ showed the lowest Ca levels. Moreover, significantly less Ca accumulation was found in ES + $ZnCl_2$ at 500 mg $kg^{-1}$, compared to that in bulk ZnO (42%) and Z-COTE HP1 (39%) at the same concentration. It is known that Ca and Zn have an antagonistic interaction (Rusjan, 2012). This suggests that the highest transport of Zn (128 mg $kg^{-1}$) to seeds from ES + $ZnCl_2$ at 500 mg $kg^{-1}$ diminished the accumulation of Ca.

**Trace minerals**

Seed accumulation of Fe, Cu, Mn, Mo, and Ni was also evaluated. Interestingly, only Fe and Mn were accumulated at significantly higher amounts in seeds from the ES, compared to the seeds from NS.

Beans are known as excellent sources of Fe, providing an average of 66.9 ± 2.4 mg $kg^{-1}$, (USDA, 2015), a lower value than that recorded in the seeds from this study (138.9 ± 16.8 mg $kg^{-1}$), which indicates good quality of the produced seeds. Since some incongruence was found in the quantification of Fe in seeds, the data have been excluded for further discussion.

Table 3.9 shows the Cu concentration in kidney bean seeds. The statistical analysis indicated a significant effect of the soil type and the Zn compound. Even when none of the treatments resulted different from their respective controls, NS seeds grown in bulk ZnO and Z-COTE at 500 mg $kg^{-1}$ accumulated ≈26% less Cu than the seeds grown in the presence of Z-COTE HP1. There is the possibility that $Cu^{2+}$ or $Cu^{+}$ ions in NS were attracted to the negative charge of Z-COTE HP1 (23.6 ± 0.9 mV), making them less available in the soil. In addition, the activation of the expression of transporters of the ZIP family (White & Broadley, 2009), which transport both Cu and Zn (Alloway, 2004), might have happened. In our previous study on kidney beans, bulk ZnO in NS showed a similar Cu reduction in stems (at concentrations of 62.5-500 mg $kg^{-1}$), and in leaves at 250 and 500 mg $kg^{-1}$ (Medina-Velo *et al.*, 2017a).

Accumulation of Mn in seeds is shown in Table 3.10 This table shows that none of the individual treatments had significant differences in the Mn content, compared with their respective controls. However, the statistical analysis indicated a significant effect of the soil type, the concentration, and the interactions soil × compound and soil × concentration. The soil effect increased the Mn content by ≈85% in the ES seeds in comparison to the seeds grown in NS.

The interaction between Zn compounds in the two soil types is illustrated in Fig. 3.9a. In the NS, the seeds exposed to all compounds had a similar Mn accumulation, while in the

Table 3.9: Copper content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control

| | | | **Natural soil** | | | **Enriched soil** |
|---|---|---|---|---|---|---|
| **Nutrient** | **Compound** | **Concentration (mg $kg^{-1}$)** | $\overline{x}$ ± **SD** | | | $\overline{x}$ ± **SD** |
| **Cu** | **Bulk ZnO** | **Control** | 8.63 ± 1.20 | | ∧ | 9.11 ± 0.97 |
| **(mg $kg^{-1}$)** | | **62.5** | 11.12 ± 1.51 | | | 8.22 ± 1.23 |
| | | **125** | 9.59 ± 2.26 | | | 9.57 ± 3.38 |
| | | **250** | 10.36 ± 2.41 | | | 8.09 ± 1.19 |
| | | **500** | 9.58 ± 1.22 | a | | 8.48 ± 1.46 |
| | **Z-COTE** | **Control** | 10.28 ± 0.44 | | ∧ | 7.80 ± 0.77 |
| | | **62.5** | 9.53 ± 1.31 | | | 7.37 ± 0.38 |
| | | **125** | 11.26 ± 2.16 | | ∧ | 8.19 ± 2.36 |
| | | **250** | 9.16 ± 0.96 | | | 7.94 ± 2.06 |
| | | **500** | 9.43 ± 1.69 | a | | 8.06 ± 0.90 |
| | **Z-COTE HP1** | **Control** | 11.19 ± 1.18 | | ∧ | 7.07 ± 0.83 |
| | | **62.5** | 9.83 ± 1.39 | | | 8.97 ± 2.72 |
| | | **125** | 9.53 ± 2.19 | | | 8.16 ± 0.46 |
| | | **250** | 9.99 ± 0.92 | | | 9.56 ± 1.95 |
| | | **500** | 12.78 ± 2.07 | b | ∧ | 8.04 ± 1.76 |
| | **$ZnCl_2$** | **Control** | 10.37 ± 1.80 | | | 8.47 ± 0.84 |
| | | **62.5** | 9.69 ± 1.67 | | | 9.63 ± 1.25 |
| | | **125** | 10.54 ± 1.59 | | | 10.65 ± 2.34 |
| | | **250** | 11.71 ± 1.70 | | | 9.54 ± 2.43 |
| | | **500** | 11.84 ± 1.02 | ab | ∧ | 9.46 ± 0.74 |

Table 3.10: Manganese (Mn), molybdenum (Mn), and nickel (Ni) content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Values are average ± standard deviation of 4 replicates. Letters represent differences between-compounds at the same concentration and within the same soil type; the symbol ∧ represents differences between-soil within the same compound, at the same concentration; and *stands for differences against the respective control

| | | | **Concentration (mg kg$^{-1}$)** | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Nutrient** | **Soil** | **Compound** | **Control** | | | **62.5 mg kg$^{-1}$** | | | **125 mg kg$^{-1}$** | | | **250 mg kg$^{-1}$** | | | **500 mg kg$^{-1}$** | | | | |
| **Mn** | **NS** | **Bulk ZnO** | ∧ 18.02 | ± | 1.47 | ∧ 17.91 | ± | 1.79 | ∧ 18.69 | ± | 1.66 | ∧ 18.36 | ± | 1.15 | ∧ 18.67 | ± | 2.30 | ab |
| **(mg kg$^{-1}$)** | | **Z-COTE** | ∧ 19.07 | ± | 1.37 | ∧ 18.00 | ± | 1.78 | ∧ 20.02 | ± | 5.09 | ∧ 17.79 | ± | 0.37 | ∧ 16.29 | ± | 2.11 | a |
| | | **Z-COTE HP1** | ∧ 18.81 | ± | 2.18 | ∧ 17.28 | ± | 0.78 | ∧ 17.47 | ± | 1.00 | ∧ 18.96 | ± | 1.80 | ∧ 18.07 | ± | 1.89 | ab |
| | | **$ZnCl_2$** | ∧ 18.48 | ± | 0.25 | ∧ 16.08 | ± | 4.66 | ∧ 20.28 | ± | 2.00 | ∧ 15.93 | ± | 2.33 | ∧ 22.18 | ± | **6.24** | **b** |
| | **ES** | **Bulk ZnO** | 34.30 | ± | 1.99 | 33.51 | ± | 1.98 | 34.25 | ± | 2.81 | 34.55 | ± | 2.09 | 37.69 | ± | 1.75 | |
| | | **Z-COTE** | 34.68 | ± | 4.28 | 33.88 | ± | 0.77 | 32.25 | ± | 1.21 | 35.47 | ± | 2.08 | 35.58 | ± | 2.38 | |
| | | **Z-COTE HP1** | 33.22 | ± | 1.51 | 33.27 | ± | 1.90 | 33.05 | ± | 0.26 | 35.71 | ± | 3.43 | 36.56 | ± | 1.44 | |
| | | **$ZnCl_2$** | 31.77 | ± | 1.37 | 31.64 | ± | 1.17 | 31.03 | ± | 1.84 | 32.97 | ± | 2.35 | 33.50 | ± | **2.04** | |
| **Mo** | **NS** | **Bulk ZnO** | 4.63 | ± | 2.07 | 7.61 | ± | 4.37 | 5.41 | ± | 6.85 | 8.41 | ± | 7.83 | 6.49 | ± | 3.24 | a |
| **(mg kg$^{-1}$)** | | **Z-COTE** | 4.07 | ± | 3.47 | 3.68 | ± | 2.14 | 8.75 | ± | 3.91 | 6.05 | ± | 2.47 | 9.29 | ± | 3.07 | ab |
| | | **Z-COTE HP1** | 7.93 | ± | 4.50 | 5.88 | ± | 2.73 | 7.30 | ± | 3.70 | 8.39 | ± | 4.69 | ∧ ***15.89** | ± | **5.60** | b |
| | | **$ZnCl_2$** | 7.37 | ± | 3.81 | 5.86 | ± | 5.48 | 4.48 | ± | 5.51 | ∧ 11.06 | ± | 3.13 | 8.21 | ± | 5.67 | a |
| | **ES** | **Bulk ZnO** | 6.69 | ± | 1.43 | 5.90 | ± | 3.80 | 6.04 | ± | 2.75 | 5.95 | ± | 1.35 | 7.29 | ± | 1.14 | |
| | | **Z-COTE** | 4.38 | ± | 1.01 | 1.82 | ± | 1.37 | 5.65 | ± | 4.38 | 5.15 | ± | 3.64 | 5.50 | ± | 1.61 | |
| | | **Z-COTE HP1** | 3.53 | ± | 2.28 | 2.52 | ± | 1.02 | 2.22 | ± | 1.28 | 4.32 | ± | 3.61 | 6.49 | ± | 4.08 | |
| | | **$ZnCl_2$** | 3.81 | ± | 0.16 | 5.60 | ± | 3.18 | 6.37 | ± | 2.84 | 3.76 | ± | 3.70 | 5.95 | ± | 0.85 | |
| **Ni** | **NS** | **Bulk ZnO** | 3.24 | ± | 0.80 | 2.70 | ± | 0.26 a | ***1.93** | ± | **0.81** a | ***1.98** | ± | **0.51** | ***1.63** | ± | **0.68** | ab |
| **(mg kg$^{-1}$)** | | **Z-COTE** | ∧ 3.84 | ± | 0.68 | ***1.78** | ± | **0.50** ab | ***1.72** | ± | **0.46** a | ***1.65** | ± | **0.31** | ***1.32** | ± | **0.38** | a |
| | | **Z-COTE HP1** | 3.17 | ± | 1.09 | ***1.56** | ± | **0.57** b | ***1.81** | ± | **0.66** a | ***1.19** | ± | **0.35** | ***1.81** | ± | **0.48** | ab |
| | | **$ZnCl_2$** | ∧ 3.95 | ± | 0.39 | ∧ 4.17 | ± | 1.06 c | ∧ 4.15 | ± | 1.48 b | ***2.27** | ± | **0.52** | ***2.73** | ± | **0.39** | b |
| | **ES** | **Bulk ZnO** | 2.91 | ± | 0.45 | 2.25 | ± | 0.24 | 1.99 | ± | 1.09 | 2.10 | ± | 0.45 | 1.75 | ± | 0.55 | |
| | | **Z-COTE** | 2.79 | ± | 0.56 | 1.70 | ± | 0.41 | ***1.48** | ± | **0.58** | ***1.42** | ± | **0.32** | 1.65 | ± | 0.55 | |
| | | **Z-COTE HP1** | 2.66 | ± | 0.60 | 2.14 | ± | 0.29 | ***1.45** | ± | **0.34** | 1.61 | ± | 0.46 | ***1.03** | ± | **0.31** | |
| | | **$ZnCl_2$** | 2.82 | ± | 0.43 | 2.83 | ± | 0.36 | 2.33 | ± | 0.35 | 2.21 | ± | 0.46 | 1.98 | ± | 0.38 | |

ES, the seeds from the $ZnCl_2$ treatment had ≈7% less Mn than the seeds from the rest of the compounds. Reductions in Mn accumulation in leaves and stems of kidney bean plants were reported after exposure to $ZnCl_2$ (Medina-Velo *et al.*, 2017a). Similarly to what has been observed with other nutrients, $ZnCl_2$ impacted the accumulation of Mn, possibly due to the increased activity in the transporters of the ZIP family, implicated in both Zn and Mn transport (Medina-Velo *et al.*, 2017a, Pittman, 2005).

The molybdenum content in seeds is shown in Table 3.10. Like Mn, the seed Mo content was significantly affected by the soil and concentrations, and the soil × compound interaction.

Seeds from the NS + Z-COTE HP1 treatment at 500 mg $kg^{-1}$ resulted in 100% more Mo than their respective controls. Molybdenum is absorbed from the soil in the form of molybdate ($MoO_4^{2-}$) (Nie *et al.*, 2014). Thus, there is the possibility of negatively charged Z-COTE HP1 upregulating the molybdate transporter 1 (MOT1) in the roots, enhancing Mo uptake (Nie *et al.*, 2014). The soil × compound interaction is shown in Fig. 3.9b. This figure shows that seeds from both soils amended with all four tested compounds accumulated similar amounts of Mo. However, there was a significant reduction of Mo in seeds grown in ES amended with Z-COTE HP1 and $ZnCl_2$, with values of ≈58% and ≈31%, respectively, lower than the seeds from the NS plants. It has been suggested that molybdenum attaches to the mineral components in acidic soils or binds to OM ligands (Wichard *et al.*, 2009), causing a strong Mo retention in the soil (Marks *et al.*, 2015), which diminishes its availability for the plant uptake.

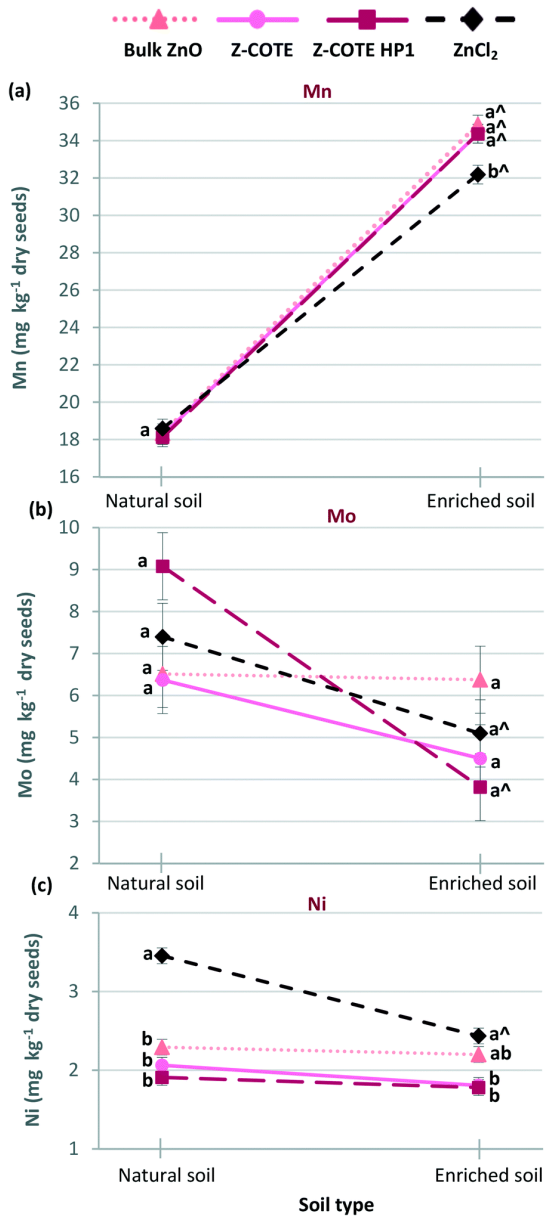


Figure 3.9: Soil × compound interaction plot of (a) manganese, (b) molybdenum, and (c) nickel content of red kidney bean seeds grown in natural soil (NS) or organic-matter enriched soil (ES) amended with bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 62.5, 125, 250, and 500 mg $kg^{-1}$. Data are average ± standard deviation of 20 replicates (concentration effect is not considered); and SE= (a) 352.485, (b) 0.819, and (c) 0.134. Letters represent differences between-compounds within the same soil type, and the symbol ∧ represents differences between-soil type within the same compound.

Nickel accumulation in seeds is shown in Table 3.10. As it can be seen, kidney bean seeds had small concentrations of Ni. However, statistically significant differences were observed in plants grown in the NS and the ES.

Seeds from NS amended with uncoated and coated ZnO NMs at all concentrations had ≈58% and ≈50%, respectively, less Ni than their controls. Similarly, bulk ZnO at 125 mg $kg^{-1}$ and above reduced the seed Ni content by ≈43%, compared with the controls; while $ZnCl_2$ showed reductions of about 37% at 250 and 500 mg $kg^{-1}$, compared with the controls. In the ES, reductions were found in seeds grown in Z-COTE at 125 (47%) and 250 mg $kg^{-1}$ (49%), Z-COTE HP1 at 125 (45%) and 500 mg $kg^{-1}$ (61%), compared with the control. It has been suggested that $Ni^{2+}$ and $Zn^{2+}$ are absorbed by plants using the same carrier site (Cataldo *et al.*, 1978); thus, a higher Zn content may have inhibited Ni absorption and accumulation in plants. Since these reductions were only observed in the ZnO NMs, it suggests an effect of the particle size (Medina-Velo *et al.*, 2017a). Finally, the soil × compound interaction (Fig. 3.9c) shows that seeds exposed to $ZnCl_2$ in the NS and ES exhibited the highest Ni amounts, but this accumulation was diminished by 30% in the ES, in comparison to that in the NS. Similar to Mo, it has been reported that Ni forms complexes with OM components (Mishra *et al.*, 2014), making it less available for the plant uptake.

## 3.4 Conclusions

This study aimed to determine the effects of surface coating of ZnO NMs in the nutritional composition of common beans cultivated in soils with different physicochemical properties. The results indicate an increase on Zn accumulation in seeds of plants grown in OM-enriched soil (38%), compared with the NS. However, exposure to Z-COTE HP1 at 125 mg $kg^{-1}$ and above, in the ES, resulted in lower seed Zn content, compared to bulk ZnO and $ZnCl_2$, suggesting that the hydrophobic coating interacted with soil components in a different way than uncoated ZnO. The data do not support the working hypothesis, that in the NS,

Zn availability for plants exposed to Z-COTE HP1 would be lower than those exposed to Z-COTE, due to the coating's hydrophobicity. However, it supports our hypothesis that no difference would be found in Zn availability in the ES, due to the presence of OM. The change in the OM (2.8% in the NS and 18% in the ES) content had a significant impact on the effect of the ZnO NMs or compounds in bean production and the nutrients of red kidney bean seeds. Another important soil factor affecting the interaction of the Zn compounds with bean plants was the pH. The reduction of pH after OM addition changed most of the minerals' mobility, by increasing their accumulation in the seeds (except Mo and Ni, which were reduced in some cases). The increase in OM also reduced the time to reach maturity ($\approx$25 days) and increased the seed production; however, Z-COTE HP1 and $ZnCl_2$ reduced relative sugar accumulation in the seeds, compared to seeds harvested from the NS.

As expected, there was an interaction of soil properties $\times$ compounds. For instance, plants exposed to bulk ZnO in ES were the last to reach maturity, while bulk ZnO and Z-COTE reduced the maturation time in the NS ($\approx$12 days), compared to Z-COTE HP1 and $ZnCl_2$.

Interestingly, and in contrast to the reported toxicity of ZnO NMs (Reddy *et al.*, 2016), seeds from Z-COTE exposed plants only showed a significant increase in Mg (in the NS at 125 mg $kg^{-1}$) and decreases in nickel (in the ES at 125 and 250 mg $kg^{-1}$) in comparison with the controls. On the other hand, Z-COTE HP1 impacted the amounts of Mo, Ni and K, in addition to the sugar content and Zn. $ZnCl_2$ was the compound that impacted the most the nutritional content of the seeds, which could be attributed to the presence of $Cl^-$ or the high availability of Zn after dissolution. The different behavior of Z-COTE and Z-COTE HP1 opens the door for further investigations to elucidate the mechanisms of action of these compounds. Overall, this investigation has shown that the effects of coated and uncoated ZnO nanomaterials on bean plants are affected by the soil conditions.

# Chapter 4

## Minimal transgenerational effect of ZnO nanomaterials on the physiology and nutrient profile of *Phaseolus vulgaris*[3]

## 4.1 Introduction

The fate of nanomaterials (NMs) incorporated into personal care products (PCPs) is still largely unknown. It has been estimated that 28-32% of such NMs are released to bodies of water (Keller *et al.*, 2014). Particles such as ZnO NMs, Z-COTE, and Z-COTE HP1 are widely used in PCPs. The Z-COTE bare ZnO NM has an amphiphilic nature and is used in water-based formulations; Z-COTE HP1, has a hydrophobic coating of triethoxycaprylylsilane and is suitable for oil-based formulations.

ZnO NMs can reach agricultural environments unintentionally when released from PCPs or intentionally as agrochemicals (Sturikova *et al.*, 2018). These NMs, and the derived ions, can also reach agricultural fields through irrigation with NM-containing water or biosolids (Rizwan *et al.*, 2016). The effects of ZnO NMs on plants and the accumulation of Zn after exposure has been studied. Physiological and biochemical effects of such nanomaterials in exposed wheat (Du *et al.*, 2011), soybean (Hernandez-Viezcas *et al.*, 2013, Peralta-Videa *et al.*, 2014), peanut (Prasad *et al.*, 2012), kidney bean (Medina-Velo *et al.*, 2017a), cucumber (Zhao *et al.*, 2013a), pea (Mukherjee *et al.*, 2016), and sorghum (Dimkpa *et al.*, 2017) have been reported. However, the residual effects of the exposure to ZnO NMs

[3]Medina-Velo, I. A., Zuverza-Mena, N., Tamez, C., Ye, Y., Hernandez-Viezcas, J. A., White, J. C., Peralta-Videa, J. R., and Gardea-Torresdey, J. L. (2017) Minimal transgenerational effect of ZnO nanomaterials on the physiology and nutrient profile of *Phaseolus vulgaris*. *ACS Sustainable Chemistry & Engineering* (submitted and under review)

across multiple plant generations (MG) remains unexplored (Servin & White, 2016). Long-term exposure to NMs may cause stress and possibly physiological adaptations (Singh & Kumar, 2018). The adaptation process can be associated with phenotypic or genotypic changes (Bicho *et al.*, 2017), or by epigenetic modifications (i.e., changes in gene function that cannot be explained by alterations in the DNA sequence) (Wong *et al.*, 2017). Several contaminants have been reported to produce epigenetic changes in plants such as *Arabidopsis thaliana* upon exposure to ZnO, $TiO_2$ , and fullerene soot (Landa *et al.*, 2012), and in *Zea mays* in response to Zn stress (Erturk *et al.*, 2015). Furthermore, transgenerational epigenetic effects can be observed in non-exposed plants from the subsequent generation if the effect on the maternal gene function prevails (Bicho *et al.*, 2017).

The assessment of plant response to NMs commonly includes determining the impact on biomass, growth, yield, bioaccumulation of NM/ions, and nutrient composition. In addition, given that NMs can interact with biological molecules, stress biomarkers such as increases in reactive oxygen species (ROS) can be used as an assessment of toxicity (Shaw *et al.*, 2017, Wong *et al.*, 2017). Plants respond to excess ROS through antioxidant defense systems by converting ROS into less damaging species (Alscher *et al.*, 2002, Sturikova *et al.*, 2018). Antioxidant enzymes include superoxide dismutase (SOD), which catalyzes the dismutation of superoxide radical ($O_2^{\cdot -}$) to $H_2O_2$ and $O_2$ (Raychaudhuri & Deng, 2000, Rico *et al.*, 2013), ascorbate peroxidase (APX), and catalase (CAT); the latter two convert $H_2O_2$ to $H_2O$.

Only a few intergenerational studies on the effects of NMs in plants are found in the literature. In *Brassica rapa*, the consecutive exposure of three generations to $CeO_2$ NMs showed higher oxidative stress and lower seed production in the later generations (Ma *et al.*, 2016). Rico *et al.* (2017) reported changes in the physiology and nutrient profile of the second generation of wheat *(Triticum aestivum)* plants exposed to $CeO_2$ NMs. The MG exposure to nano-$CeO_2$ produced grains with lower Mn, Ca, K, Mg, and P, compared with single exposure (Rico *et al.*, 2017). However, to the authors knowledge, studies on the residual effects of the exposure to Z-COTE and Z-COTE HP1 have not been reported.

Hence, the objective of this study was to measure the nutrient profile and physiological status in second generation bean seeds (S2). The seeds from the first generation (S1) were obtained from plants cultivated in soil amended with either with Z-COTE, Z-COTE HP1, bulk ZnO, or $ZnCl_2$ (Medina-Velo *et al.*, 2017c). Then, S1 seeds were grown in clean soil and S2 were analyzed. Spectroscopic and biochemical techniques were used to evaluate the residual effects of the exposure to Zn nanomaterials or compounds by measurement of Zn accumulation, nutritional profiles, and antioxidant activity in the S2 seeds.

## 4.2 Experimental section

### 4.2.1 Nanomaterials, compounds, and soil

Bulk ZnO ($\geq$ 1000 nm, ACS reagent $\geq$ 99% purity) was purchased from Sigma Aldrich; Z-COTE (286 $\pm$ 2 nm), Z-COTE HP1 (276 $\pm$ 7 nm) were purchased from BASF; and ionic $ZnCl_2$ (ACS reagent $\geq$ 97% purity) was purchased from Acros Organics. Nanomaterial characterization has been previously reported (Medina-Velo *et al.*, 2017a). Maternal bean seeds *(Phaseolus vulgaris* var red hawk) (S0) were supplied by Dr. James Kelly from Michigan State University and stored at 4°C until experimentation (Majumdar *et al.*, 2014). Medium loam soil was collected from an agricultural field in Texas (N 31° 40.489, W 106° 17.198, elevation: 1115 MASL) and commercial Miracle Grow potting mix was purchased from a local store.(Majumdar *et al.*, 2016) Both soils were sieved through an 8 mm mesh to exclude larger materials and a mixture for plant growth was prepared by mixing 250 g of medium loam soil and 250 g of potting mix (50:50 %wt).

### 4.2.2 Maternal seed exposure and growth

Four replicates of powders of bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ were weighed to achieve concentrations of 125, 250 and 500 mg $kg^{-1}$ soil (compound based) and were suspended in 50 mL of deionized water (DW), as previously described (Majumdar *et al.*,

2015). Bulk ZnO and Z-COTE suspensions were sonicated in a water bath (Crest Ultrasonics, Trenton, NJ) for 30 min at 25°C and at an intensity of 180 watts. Without sonication, $ZnCl_2$ was dissolved and Z-COTE HP1 was manually mixed into solution. The suspensions/solutions were added to the soil, mixed with a hand cultivator to optimize homogeneity, transferred into plastic pots (12.5 cm diameter × 14 cm height) and left at room temperature for 24 h. Four replicates of soil watered with DW were used as controls for each compound. The S0 bean seeds were washed with 2% NaClO, rinsed three times with DW for disinfecting purposes, and were soaked in DW to facilitate germination. After 24 h, five S0 seeds were planted in each pot at 2.5 cm depth and were watered with 50 mL of DW; the pots were transferred to a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) with 14 h photoperiod (340 $\mu$mole $m^{-2}$ $s^{-1}$), 25/20 °C day/night temperature and 65-70% relative humidity). The plants were watered with 50 mL of DW until germination, then with 100 mL DW until S1 seeds were mature (≈15% humidity. Although the experiment was set in a growth chamber with uniform conditions, the pots were randomly rotated every 14 days to reduce any possible differences in illumination. At maturity (87 ± 11 days) (Medina-Velo *et al.*, 2017c), pods were cut from the calyx, including the pedicel; the S1 seeds were removed from the pods, placed in plastic bags, and stored at 4 °C.

### 4.2.3 Growth of second generation plants

The S1 seeds were disinfected with 2% NaClO, rinsed 3 times with DW, and soaked in DW for 24 h. Three seeds from each treatment/replicate were planted in plastic pots at 2.5 cm depth with 500 g of uncontaminated soil mix watered with 50 mL DW. The pots were transferred to an environmental growth chamber with the same conditions used in the first generation. All seeded pots were watered with 50 mL DW until germination, then with 100 mL until maturity, and were randomly rotated every 14 days. Forty-five days after planting, when all pods were fully developed, one pod from each plant/replicate was collected and immature S2 seeds were obtained for enzyme extractions. Plants were then

allowed to grow until maturity; pods were then collected, and the weight and number of S2 seed-containing pods was recorded. The S2 seeds were removed from the pods and the total number of seeds was recorded, along with the weight of the pods and seeds. The S2 seeds were placed in paper envelopes and oven dried at 70 °C for 72 h.

### 4.2.4 Antioxidant enzymatic activity in young seeds

One immature (45 days after planting) pod from each plant/replicate was collected and one S2 seed was isolated from each pod. The seeds from each replicate were mixed and two sub-samples of ≈1 g were ground in liquid nitrogen and extracted with 10 mL of 0.1 M phosphate buffer (pH 7.8) using a mortar and pestle. The homogenate was centrifuged at 16 000 × g at 4 °C for 15 min (Sorvall Legend X1R, Thermo Scientific, Waltham, MA) (Bailly *et al.*, 2001). The supernatant was distributed into five Eppendorf tubes, frozen in liquid nitrogen, and stored at -80 °C until analysis. The enzyme analyses were performed by measuring absorbance in a quartz cuvette using a UV/Vis Spectrometer (single-beam mode, Perkin-Elmer Lambda 14, Uberlingen, Germany). The APX (EC 1.11.1.11) activity was determined according to Bailly *et al.* (2001) and Nakano & Asada (1981) by the decrease in the absorbance of $H_2O_2$ at 290 nm during a 2 min interval. The reaction mix contained 50 $\mu$L of sample, 285 $\mu$L of 0.5 mM ascorbic acid, and 665 $\mu$L of 0.4 mM $H_2O_2$ . The CAT (EC 1.11.1.6) activity was determined as reported by Bailly *et al.* (1996). Sixty seven $\mu$L of extract were mixed with 933 $\mu$L of 3.125 mM $H_2O_2$ in 50 mM phosphate buffer (pH 7.0). Changes in absorbance were recorded at 240 nm during a 3 min interval. The APX and CAT activities were expressed as nmol $H_2O_2$ decomposed (g fresh seed)$^{-1}$ min$^{-1}$. The SOD (EC 1.15.11) (Bailly *et al.*, 1996, Beyer Jr & Fridovich, 1987, Giannopolitis & Ries, 1977, Rico *et al.*, 2013, Zhang *et al.*, 2016) assay contained 450 $\mu$L of 500 $\mu$M nitroblue tetrazolium (NBT), 500 $\mu$L of 78 mM L-methionine, 200 $\mu$L of 1.5 mM EDTA, 300 $\mu$L of 0.02 mM riboflavin, 1500 $\mu$L of 100 mM potassium phosphate buffer (pH 7.8) and 50 $\mu$L of enzyme extract. The SOD activity was estimated by measuring inhibition of the photochemical reduction of NBT by the enzyme extract. The reaction mixture was placed

in a glass test tube and illuminated with a fluorescent light bulb in a closed box during 15 min, and then absorbance ($A_1$) was measured at 560 nm. Non-illuminated tubes with the reaction mix without seed served as blanks, and absorbance after illumination was recorded ($A_0$). One unit of SOD is defined as the enzymatic activity that causes 50% inhibition of the assay reaction; the % inhibition of NBT reduction by SOD was calculated by ($A_0$ - $A_1$)/$A_0$.

### 4.2.5 Quantification of total sugar, starch, and protein

Sugar and starch were extracted from 0.1 g of dry seed as described by Verma & Dubey (2001). Total sugar and starch were quantified by the phenol-sulfuric acid method in a microplate format(Masuko *et al.*, 2005) using a Spectra Max 190 Microplate Reader (Molecular Devices, San Jose, CA). The protein content was analyzed in a LECO FP628 Nitrogen Determinator (Saint Joseph, MI) following the nitrogen combustion method as described by Chang (2014). LECO reference materials of wheat flour and EDTA were used for quality control and quality assurance (QA/QC) during protein determination.

### 4.2.6 Essential element quantification via ICP-OES

Quantification of major (Ca, K, Mg, P, S) and trace (B, Cu, Fe, Mo, Mn, Ni, and Zn) minerals in S2 seeds was done with dry powdered samples. Samples of 0.1-0.2 g were pre-digested with 3 mL of 70% $HNO_3$ for 45 min at room temperature. For digestion, the samples were heated on a hot block digestion system (SCP Science, Champlain, NY) at 115 °C for 45 min followed by dilution to 50 mL with ultra-pure water (UPW, 18.2 MΩ $cm^1$). The elemental composition was measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES, iCAP 6500, Thermo Fisher Scientific, Waltham, MA). The ICP-OES parameters were as follows: nebulizer flow, 0.50 L/min; power, 1,150 W; peristaltic pump rate, 45 rpm; and flush time, 45 s. For QC of the ICP-OES readings, a multi-elemental standard solution of 1 mg/L was analyzed every 12 samples and Y was

used as internal standard. The National Institute of Standards and Technology (NIST) standard reference material 1570a (spinach leaves) was used to validate the digestion and analytical method; a Zn analyte recovery of 93% was achieved.

### 4.2.7 Statistical analysis

The Statistical Package for the Social Sciences 22 (SPSS, Chicago, IL, USA) was utilized for one-way ANOVA tests to evaluate the experimental variance. Differences between treatments were evaluated with the multi comparison Tukeys HSD test at a $p$-value of 0.05. Data presented are means of specified number of replicates.

## 4.3 Results and discussion

### 4.3.1 Days to reach maturity, number of plants, and yield

Table 4.1 shows the data for number of days to reach maturity, number of germinated plants, and pod/seed production of the second-generation bean plants; none of the treatments affected these parameters relative to the control. Plants from the second generation matured at 107 ± 5 days, while plants from the first generation lasted 87 ± 11. Additionally, all S2 seeds pre-exposed to Z-COTE HP1 successfully germinated (mean= 3.0 ± 0.0).

There was no transgenerational effect of treatments in the number and weight of seeds (Table 4.1), However, the number of pods in the second generation was reduced (45%, $p \leq 0.05$) by $ZnCl_2$ at 250 mg $kg^{-1}$, compared with bulk ZnO at the same concentration. The treatments did not affect the S2 seed weight; however, the S1 seed weight from our previous study was decreased by 74% under exposure to $ZnCl_2$ at 250 mg $kg^{-1}$, compared with bulk ZnO at the same concentration (Medina-Velo *et al.*, 2017c). A negative effect in production caused by $ZnCl_2$ has been attributed to the formation of choline chloride (Chandler & Dean, 1994), possibly formed by $Cl^-$ ions released from $ZnCl_2$ or to a toxic effect of $Cl^-$ anion that

has been reported to decrease plant growth and yield (Hajrasuliha, 1980, Tavakkoli *et al.*, 2010). Overall, the results suggest minimal physiological impact on the plants from parental pre-exposure to the nanomaterials; the effects of $ZnCl_2$ were similarly inconsequential at genetic level since no impact was evident in the second generation of plants.

Table 4.1: Time to reach maturity, number of plants, pods, and seeds by the second generation of bean plants cultivated in nanoparticle-free soil. Maternal plants were exposed to bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 125, 250, and 500 mg $kg^{-1}$. Values are means of 4 replicates for Zn compounds and 16 for controls. Letters represent statistically significant differences between means of the different compounds at the same concentration ($p \leq 0.05$). The treatment concentrations refer to S1 plants.

| | Concentration | | | Pods | | Seeds | |
|---|---|---|---|---|---|---|---|
| **Compound** | **(mg $kg^{-1}$)** | **Days (#)** | **Plants (#)** | **Number (#)** | **Weight (g)** | **Number (#)** | **Weight (g)** |
| *** | Control | 102.5 ± 4.1 | 2.8 ± 0.1 | 8.0 ± 0.5 | 12.8 ± 0.6 | 20.8 ± 1.1 | 8.9 ± 0.5 |
| **Bulk ZnO** | 125 | 105.0 ± 3.0 | 2.3 ± 0.5 | 7.8 ± 0.8 | 11.9 ± 1.7 | 17.5 ± 1.6 | 8.2 ± 1.3 |
| | 250 | 111.5 ± 6.6 | 3.0 ± 0.0 | 11.0 ± 0.91a | 15.6 ± 1.3 | 23.8 ± 2.0 | 10.8 ± 1.0 |
| | 500 | 97.3 ± 9.0 | 3.0 ± 0.0 | 9.0 ± 1.1 | 13.1 ± 2.2 | 21.3 ± 4.1 | 8.6 ± 1.7 |
| **Z-COTE** | 125 | 110.5 ± 5.2 | 2.8 ± 0.3 | 8.8 ± 1.1 | 13.2 ± 1.1 | 20.3 ± 2.4 | 9.0 ± 0.7 |
| | 250 | 114.8 ± 5.8 | 3.0 ± 0.0 | 10.0 ± 1.08ab | 13.9 ± 2.3 | 22.3 ± 3.5 | 9.6 ± 1.8 |
| | 500 | 108.3 ± 2.7 | 2.5 ± 0.3 | 8.3 ± 0.8 | 14.2 ± 1.5 | 21.3 ± 1.9 | 9.8 ± 1.0 |
| **Z-COTE HP1** | 125 | 96.0 ± 10.1 | 3.0 ± 0.0 | 7.3 ± 0.3 | 11.0 ± 0.9 | 16.8 ± 1.8 | 7.2 ± 0.7 |
| | 250 | 109.8 ± 1.8 | 3.0 ± 0.0 | 8.5 ± 0.29ab | 14.5 ± 1.0 | 22.0 ± 2.8 | 10.3 ± 0.8 |
| | 500 | 106.5 ± 1.5 | 3.0 ± 0.0 | 9.8 ± 1.5 | 15.8 ± 2.1 | 23.3 ± 2.5 | 10.6 ± 1.5 |
| **$ZnCl_2$** | 125 | 107.8 ± 7.1 | 2.0 ± 0.4 | 7.3 ± 0.8 | 13.4 ± 2.3 | 20.5 ± 2.8 | 9.5 ± 1.8 |
| | 250 | 109.8 ± 1.8 | 3.0 ± 0.0 | 6.0 ± 1.08b | 9.4 ± 2.3 | 13.8 ± 3.0 | 6.5 ± 1.8 |
| | 500 | 115.3 ± 5.2 | 2.8 ± 0.3 | 7.8 ± 1.1 | 14.0 ± 1.9 | 22.0 ± 2.6 | 9.6 ± 1.3 |

### 4.3.2 Total sugar, starch and protein

Quantification of sugar, starch, and protein content in the S2 seeds is shown in Table 4.2. Similar to the growth/production parameters, none of the treatments significantly affected sugar, starch, and protein content, compared with the control, which had 4.8%, 40.8%, and 22.9% (g/100 g), respectively. The values of protein and sugar are within the standard reference for red kidney bean (sugar- 2.10% and protein- 22.53%) according to

the USDA.USDA (2015) These values are an indicative of healthy seeds. However, parental exposure to $ZnCl_2$ reduced S2 seed sugar content by 27% at 500 mg $kg^{-1}$, comparison to Z-COTE HP1 at the same concentration. In addition, in S2 from the Z-COTE HP1 treatment at 500 mg $kg^{-1}$, the starch content trended higher (49.5%) than control (40.8%), although the difference was not statistically significant. Our previous study showed that exposure to $ZnCl_2$ at 125 and 250 mg $kg^{-1}$ significantly increased the relative sugar content (148% and 117%, respectively) in S1 compared with control seeds (Medina-Velo *et al.*, 2017c). Also in S1, the 500 mg $kg^{-1}$ Z-COTE HP1 and $ZnCl_2$ treatments resulted in similar amounts of sugar, and regardless of concentration, the relative sugar content in seeds exposed to $ZnCl_2$ was higher in comparison to the rest of the compounds. The enhanced sugar accumulation in S1 was attributed to a plant response to osmotic stress (Sturikova *et al.*, 2018) caused by the presence of $Cl^-$(Medina-Velo *et al.*, 2017c); once the stress was removed, the sugar concentrations returned to the normal levels.

Table 4.2: Sugar, starch, and protein content in second generation bean seeds cultivated from plants grown in nanoparticle-free soil. Maternal plants were exposed to bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 125, 250, and 500 mg $kg^{-1}$. Values are means of 4 replicates for Zn compounds and 16 for controls. Letters represent statistically significant differences between means of the different compounds at the same concentration ($p \leq 0.05$). The treatment concentrations refer to S1 plants.

| Compound | Concentration (mg $kg^{-1}$) | Sugar (%) | Starch (%) | Protein (%) |
|---|---|---|---|---|
| *** | Control | 4.8 ± 0.2ab | 40.8 ± 4.0 | 22.9 ± 0.4 |
| Bulk ZnO | 125 | 5.3 ± 0.4ab | 33.5 ± 5.0 | 23.6 ± 0.3 |
| | 250 | 5.1 ± 0.3ab | 56.6 ± 5.0 | 23.2 ± 0.9 |
| | 500 | 4.3 ± 0.1ab | 41.5 ± 8.9 | 22.2 ± 0.6 |
| Z-COTE | 125 | 5.3 ± 0.3ab | 47.4 ± 10.2 | 22.8 ± 1.1 |
| | 250 | 4.4 ± 0.3ab | 48.5 ± 14.9 | 23.4 ± 1.0 |
| | 500 | 5.1 ± 0.3ab | 45.7 ± 6.8 | 21.4 ± 3.6 |
| Z-COTE HP1 | 125 | 4.5 ± 0.5ab | 34.4 ± 2.6 | 22.2 ± 1.4 |
| | 250 | 5.2 ± 0.2ab | 46.8 ± 6.6 | 23.0 ± 0.5 |
| | 500 | 5.5 ± 0.1b | 49.5 ± 7.5 | 22.3 ± 1.1 |
| $ZnCl_2$ | 125 | 5.6 ± 0.3b | 40.6 ± 2.7 | 22.4 ± 0.9 |
| | 250 | 5.4 ± 0.4ab | 50.6 ± 7.6 | 21.6 ± 1.0 |
| | 500 | 4.0 ± 0.1a | 35.9 ± 1.7 | 21.0 ± 0.6 |

### 4.3.3 Zinc accumulation

In our previous study, all materials at all concentrations increased S1 Zn content in a dose-dependent fashion with values up to 147% higher than control seeds (Medina-Velo *et al.*, 2017c). However, the accumulation of Zn in S2 seeds was not significantly affected in comparison to control (Figure 4.1). Interestingly, a comparison of the residual effects across compounds at the same concentration does show differences between bulk ZnO and $ZnCl_2$. The S2 seeds harvested from S1 plants exposed to bulk ZnO at 500 mg $kg^{-1}$ had 56% more Zn than seeds from plants exposed to $ZnCl_2$ at the same concentration. The

reasons for this result are unknown. In S1 seeds, bulk ZnO and $ZnCl_2$, at 500 mg $kg^{-1}$, resulted in similar amount of Zn; thus, the Zn content in S2 cannot be due to a higher reserve of Zn. An additional study is needed to understand this difference in bulk and ionic exposure and effect.

The fact that ZnO NMs allows Zn accumulation in seeds, without transgenerational effects, represents a viable scenario for their use in Zn-deficient soils (Cakmak *et al.*, 2017). This represents a possibility to fight Zn deficiency in humans.

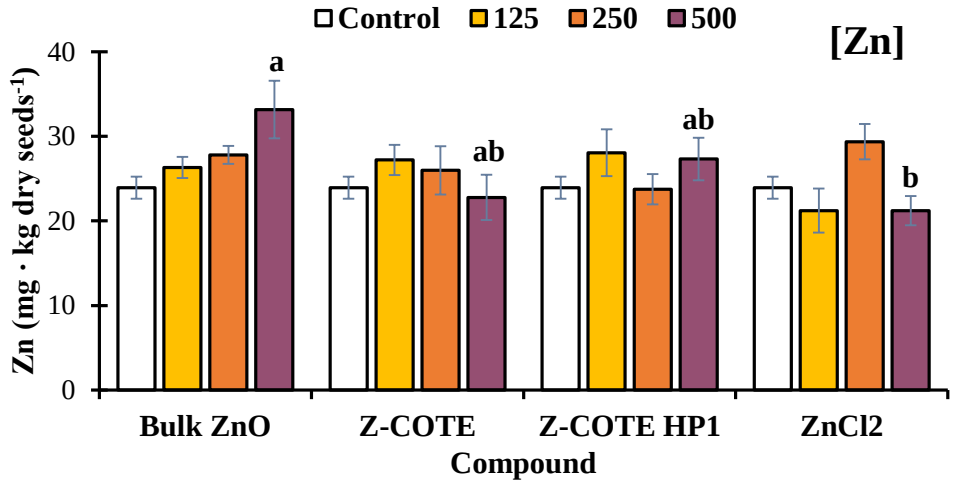


Figure 4.1: Zinc content in second generation bean seeds cultivated from plants grown in nanoparticle-free soil. Maternal plants were exposed to bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 125, 250, and 500 mg $kg^{-1}$. Values are mean ± SE of 4 replicates for Zn compounds and 14 for controls. Letters represent statistically significant differences between means of the different compounds at the same concentration ($p \leq 0.05$). The treatment concentrations refer to S1 plants.

### 4.3.4 Essential elements

In addition to Zn, major (Ca, Mg, K, P, S) and trace minerals (B, Cu, Fe, Mo, Mn, and Ni) were analyzed in S2 seeds. All essential elements in S2 except Ca and Ni were unaffected by

parental S1 exposure (Figure 4.3). The accumulation of K, P, Mg, and Fe in S2 are within the normal values as reported by the USDA (2015) (K= 13,590.0 mg $kg^{-1}$, P= 4,060.0 mg $kg^{-1}$, Mg=1,380.0 mg $kg^{-1}$, and Fe= 66.9 mg $kg^{-1}$); unfortunately, the remaining elements listed on Figure 2 are not reported by the USDA.

The accumulation of Ca and Ni was significantly affected in seeds of the second generation (Figure 3). Parental exposure to bulk ZnO at 500 mg $kg^{-1}$ increased Ca accumulation in S2 by 51% compared with the control seeds. In S1, we reported that bulk ZnO showed the highest amount of Ca (2,384 mg $kg^{-1}$) across all treatments/concentrations (Medina-Velo *et al.*, 2017c). It is possible that such Ca levels in S1 caused epigenetic effects that promoted the formation of more $Ca^{2+}$-permeable cation channels, which are responsible for Ca delivery to the plant tissues (Bicho *et al.*, 2017, White & Broadley, 2009).

Alternatively, both ZnO nanoparticles reduced Ni in S2. Z-COTE at 500 mg $kg^{-1}$ reduced S2 Ni by 60%, while Z-COTE HP1 at 125 and 500 mg $kg^{-1}$ reduced Ni 41% and 74%, respectively, compared with control seeds. Similar reductions in Ni uptake by ZnO NMs exposure were reported in S1 seeds at exposures of 125, 250, and 500 mg $kg^{-1}$. This finding was found to be nanoscale-specific,(Medina-Velo *et al.*, 2017c) since the Ni reductions were observed only with NM exposure and not the conventional microsized compounds. It has been reported that $Ni^{2+}$ and $Zn^{2+}$ are transported by the same carrier protein system (Cataldo *et al.*, 1978), which explains the Ni reductions in S1 seeds and suggests the influence of the Zn particle size in the mechanisms of Ni absorption. Importantly, the residual effect of ZnO NMs on S2 seeds suggests genetic or epigenetic changes in the transport mechanisms of Ni. Zinc accumulation produces hypomethylation of certain gene regions in *Z. mays*, which produces epigenetic regulation such as the expression of stress-related genes (Erturk *et al.*, 2015). Nickel is an essential mineral for humans; its deficiency can disturb the adsorption of Ca into the bones, which further disturbs Zn metabolism (Anke *et al.*, 1984). Although the metabolism of Ni in the human body is clearly influenced by Ca and Zn, such mechanisms are not fully understood in plants. Additional studies at the molecular level are needed to explain the mechanisms by which ZnO exposure alters Ni

accumulation in subsequent generations.

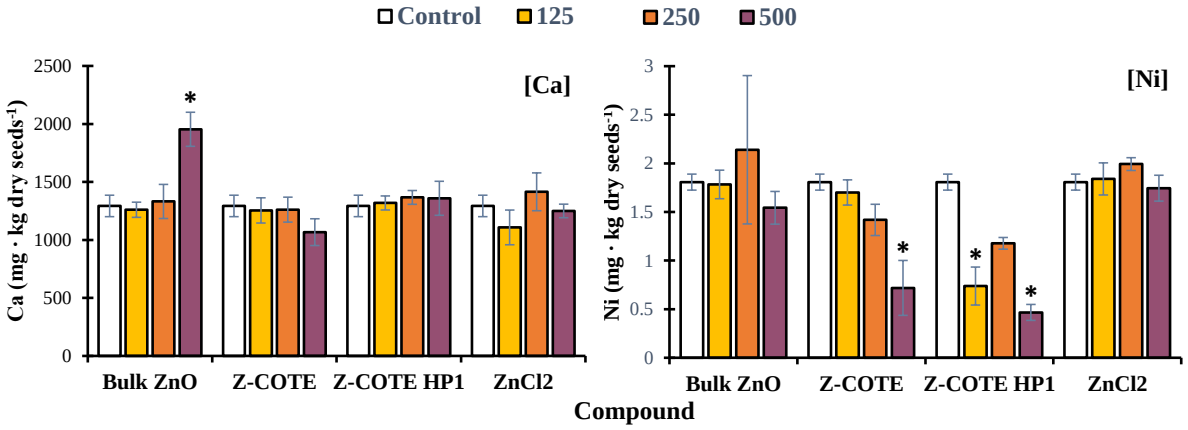


Figure 4.2: Ca and Ni content in bean seeds cultivated from plants grown in nanoparticle-free soil. Maternal plants were exposed to bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 125, 250, and 500 mg $kg^{-1}$. Values are mean ± SE of 4 replicates for Zn compounds and 14 for controls. The symbol * represents statistically significant differences between means of the different treatments and the control ($p \leq 0.05$). The treatment concentrations refer to S1 plants

| Mineral | Compound | Concentration (mg·kg dry seeds$^{-1}$) | | | |
|---|---|---|---|---|---|
| | | *Cntrl* | *125* | *250* | *500* |
| K | Bulk ZnO | 15772 | 15913 | 16173 | 16817 |
| | Z-COTE | 15772 | 16925 | 16468 | 15506 |
| | Z-COTE HP1 | 15772 | 16773 | 15598 | 16650 |
| | $ZnCl_2$ | 15772 | 15539 | 17353 | 15834 |
| P | Bulk ZnO | 4813 | 5090 | 4975 | 5222 |
| | Z-COTE | 4813 | 5282 | 5070 | 4605 |
| | Z-COTE HP1 | 4813 | 5276 | 4500 | 4950 |
| | $ZnCl_2$ | 4813 | 4423 | 5690 | 4554 |
| S | Bulk ZnO | 2325 | 2323 | 2391 | 2459 |
| | Z-COTE | 2325 | 2298 | 2279 | 2186 |
| | Z-COTE HP1 | 2325 | 2266 | 2268 | 2412 |
| | $ZnCl_2$ | 2325 | 2249 | 2434 | 2162 |
| Mg | Bulk ZnO | 1812 | 1861 | 1849 | 1996 |
| | Z-COTE | 1812 | 1848 | 1818 | 1766 |
| | Z-COTE HP1 | 1812 | 1817 | 1788 | 1814 |
| | $ZnCl_2$ | 1812 | 1805 | 1942 | 1779 |
| Fe | Bulk ZnO | 67.6 | 65.5 | 66.1 | 81.9 |
| | Z-COTE | 67.6 | 59.8 | 63.7 | 56.0 |
| | Z-COTE HP1 | 67.6 | 70.7 | 49.7 | 68.8 |
| | $ZnCl_2$ | 67.6 | 62.8 | 68.8 | 56.2 |
| Mn | Bulk ZnO | 15.3 | 15.3 | 15.2 | 16.5 |
| | Z-COTE | 15.3 | 14.1 | 16.6 | 14.3 |
| | Z-COTE HP1 | 15.3 | 15.2 | 13.8 | 14.5 |
| | $ZnCl_2$ | 15.3 | 14.9 | 16.4 | 13.8 |
| B | Bulk ZnO | 8.9 | 10.1 | 10.3 | 10.6 |
| | Z-COTE | 8.9 | 9.1 | 9.0 | 9.3 |
| | Z-COTE HP1 | 8.9 | 8.4 | 8.6 | 8.9 |
| | $ZnCl_2$ | 8.9 | 8.6 | 9.9 | 8.7 |
| Mo | Bulk ZnO | 5.1 | 5.7 | 4.6 | 6.0 |
| | Z-COTE | 5.1 | 5.1 | 5.0 | 4.7 |
| | Z-COTE HP1 | 5.1 | 5.4 | 4.5 | 4.6 |
| | $ZnCl_2$ | 5.1 | 4.7 | 6.8 | 5.4 |
| Cu | Bulk ZnO | 4.8 | 5.3 | 5.4 | 6.2 |
| | Z-COTE | 4.8 | 5.6 | 5.2 | 4.3 |
| | Z-COTE HP1 | 4.8 | 4.9 | 4.5 | 4.7 |
| | ZnCl2 | 4.8 | 4.1 | 6.0 | 3.9 |

Figure 4.3: Mineral elements that remained unaffected in bean seeds cultivated from plants grown in nanoparticle-free soil. Maternal plants were exposed to bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 125, 250, and 500 mg $kg^{-1}$. Values are means of 4 replicates for Zn compounds and 14 for controls. The lighter color represents the lowest accumulation (bottom value) and the darkest color indicates the highest accumulation (top value) for each element. The treatment concentrations refer to S1 plants.

### 4.3.5 Activity of antioxidant enzymes

The activity of CAT, APX, and SOD is shown in Figure 4.4. As evident in Figure 4.4a and b, none of the treatments significantly affected levels of the stress enzymes APX and CAT relative to the controls. However, seeds from Z-COTE HP1 at 125 mg $kg^{-1}$ showed increased APX activity relative to seeds from bulk ZnO at the same concentration. Mukherjee *et al.* (2014) reported significant increases in CAT and APX activities in leaves of pea plants upon exposure to 125, 250, and 500 mg $kg^{-1}$ of uncoated ZnO NMs. Figure 4a shows a slight decrease on APX of S2 seeds from the bulk ZnO at all concentrations, with similar decreases found in roots and leaves of pea plants grown with bulk ZnO at 125, 250, and 500 mg $kg^{-1}$.(Mukherjee *et al.*, 2014) Figure BLAb shows a decreasing trend in CAT in S2 seeds as the concentration of $ZnCl_2$ increases. Negative effects from $ZnCl_2$ were reported in S1 plants (Medina-Velo *et al.*, 2017a) and seeds (Medina-Velo *et al.*, 2017c). Moreover, salt stress reduced CAT activity in *Lupinus termis* cultivated with 150 mM NaCl (Latef *et al.*, 2017).

However, to the best of the authors knowledge, there are no reports on the activity of antioxidant enzymes in grains from plants exposed to ZnO NMs. Although the number of studies with coated NMs in plants is limited, it has been reported that in general, surface-modified NMs have different effects in plants than the pristine homologs, often affecting parameters such as metal uptake and accumulation, nutritional quality, root growth, and ROS production (Medina-Velo *et al.*, 2017b). For instance, aminopropyltriethoxysilane-coated ZnO NMs increased the biomass of green pea tissues (root, stem and leaf) and the Zn concentration in stems and leaves (Mukherjee *et al.*, 2016). In S1 seeds from our previous study, Z-COTE HP1 at 125 mg $kg^{-1}$ was the only compound/concentrations that did not increase Zn accumulation in comparison to the control. It is possible that the 1.6-fold increase in APX activity of Z-COTE HP1 in comparison to bulk ZnO seeds could be a residual effect somehow related to the different accumulation of Zn in S1 seeds from these treatments.

Superoxide dismutases are the first line of defense against ROS in the cell (Alscher *et al.*,

2002). As evident in Figure 4c, $ZnCl_2$ treatment at 500 mg $kg^{-1}$ significantly increased in SOD activity S2 compared with the control seeds. Inzé & Van Montagu (1995) stated that SOD genes are differentially regulated throughout plant development as a dynamic response to stress conditions. Increases in SOD transcript levels are caused by a rapid turnover of SOD proteins that activates the gene expression of SOD to meet the subsequent cellular demand (Inzé & Van Montagu, 1995). Although there are no reports on the antioxidant activity in bean seeds exposed to NMs, there is a possibility that the toxic effect caused by the Cl- ions in the $ZnCl_2$ exposure (reported in our previous studies) (Medina-Velo *et al.*, 2017a,c) caused stress in the first generation of plants that activated the gene expression of SOD, which subsequently manifested in the S2 seeds.

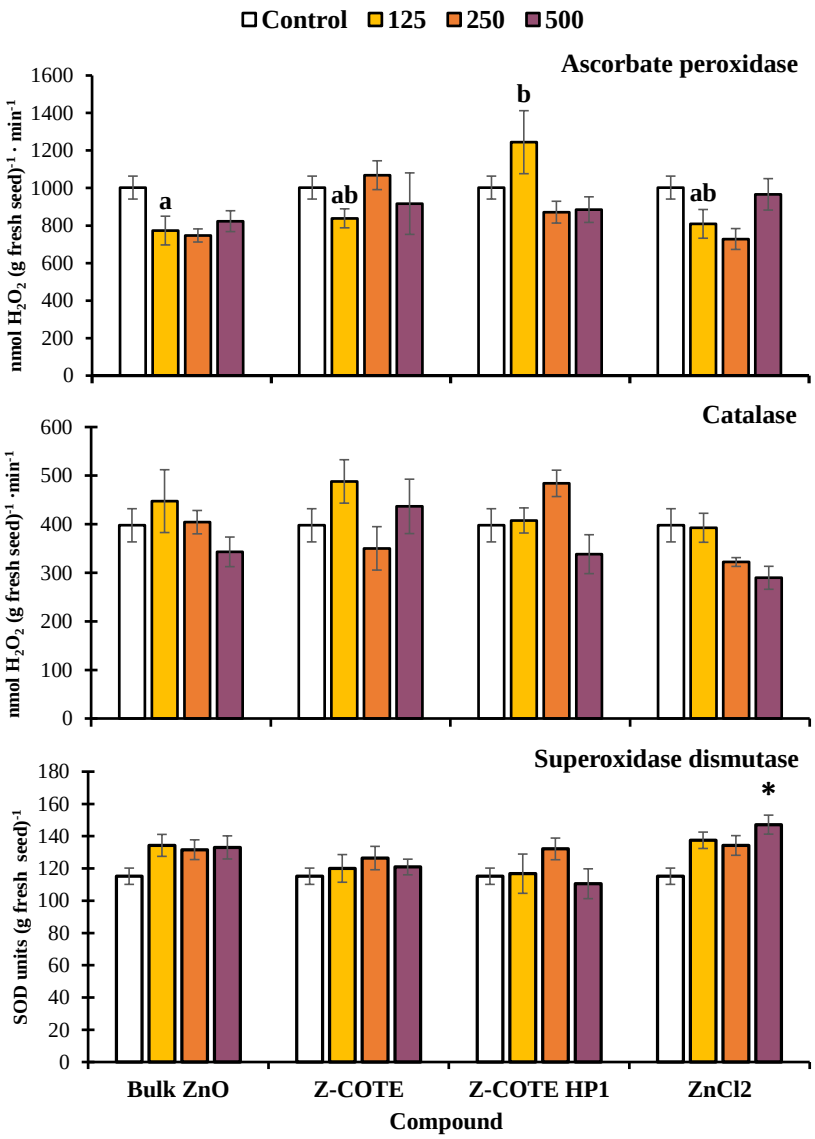


Figure 4.4: Antioxidant activity of (a) ascorbate peroxidase, (b) catalase and, (c) superoxide dismutase in bean seeds cultivated from plants grown in nanoparticle-free soil. Maternal plants were exposed to bulk ZnO, Z-COTE, Z-COTE HP1, and $ZnCl_2$ at 0 (control), 125, 250, and 500 mg kg$^{-1}$. Values are mean ± SE of 8 replicates for Zn compounds and 32 for controls. Letters represent statistically significant differences between means of the different compounds at the same concentration and the symbol * represents statistically significant differences between means of the different treatments and the control ($p \leq 0.05$). The treatment concentrations refer to S1 plants.

## 4.4 Conclusions

This study provides evidence that the residual transgenerational effects of parental ZnO NMs exposure were minimal for bean seeds subsequently cultivated in nanoparticle-free soil. Seed production and the time to reach maturity were unaffected. Seeds collected from maternal plants exposed to ZnO NMs, bulk ZnO, and $ZnCl_2$ and yielded S2 seed retained their nutritional quality, including protein, sugar, and starch content. In addition, levels of the majority of essential elements were unaffected, the exception being Ni and Ca. Nickel was reduced both in the first and second generation of seeds after exposure to ZnO NMs; the highest concentration of bulk ZnO had a residual effect that increased the accumulation of Ca in S2 seeds.

The mechanisms for these effects remain unknown, although the potential for contaminant induced genetic or epigenetic changes in the exposed parent is possible and worthy of additional investigation. While the exposure of ZnO NMs did not yield a residual negative effect in antioxidant enzyme activity, the ionic form ($ZnCl_2$) resulted in increased SOD content in S2 seeds, also possibly caused by genetic changes upon parental exposure. Further studies at the molecular and genetic level will provide necessary insight into understanding the transgenerational residual impacts of ZnO NMs but in general, this work suggests minimal consequences of the possible incorporation of ZnO NMs into Zn-deficient soils as an agricultural amendment.

# Chapter 5

## Conclusions

The present doctoral research was conducted to understand the impact of two commercially available ZnO nanomaterials (NMs) (Z-COTE and Z-COTE HP1) in the development and yield of red hawk kidney bean (*Phaseolus vulgaris*). This study provides substantial information on the effects that Z-COTE and Z-COTE HP1 have in the plant physiology and nutrition, as well as in seed quality and production. The NMs responses were also compared to the bulk ZnO and ionic $ZnCl_2$.

The first objective of this research was to evaluate the effects of Z-COTE and Z-COTE HP1 in the development of soil-grown bean plants. Results from Stage I showed that Z-COTE HP1 promoted root growth, while no effects were found for Z-COTE. On the other hand, $ZnCl_2$ at 500 mg $kg^{-1}$ significantly diminished leaf length, fresh and dry weight of leaves, and relative chlorophyll content, suggesting that the ionic form was more toxic than the NMs. The toxic effect of $ZnCl_2$ was attributed to the $Cl^-$ ion, which promotes saline stress.

Zinc accumulation in plant tissues was differentially affected by the tested compounds. Uncoated ZnO, at all concentrations, and coated ZnO at 250 and 500 mg $kg^{-1}$, increased Zn in nodules, while bulk ZnO reduced it at all concentrations. Accumulation of Zn in roots and stems was also enhanced after the exposure to Zn NMs and compounds. However, the surface coating of Z-COTE HP1 produced a different effect in the accumulation of Zn in the leaves and pods. At 500 mg $kg^{-1}$, leaves from plants grown with uncoated ZnO NMs resulted in significantly more Zn, compared with coated ZnO. Furthermore, at the highest tested concentration (500 mg $kg^{-1}$), all compounds, with exception of coated Z-COTE HP1, increased pod Zn by up to 71% in comparison to control.

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). $ZnCl_2$ affected nutrients in roots, stems and leaves, mainly increasing them. None of the treatments affected the concentration of essential elements in pods, except Zn that was significantly increased (up to 71%) by bulk ZnO at 125 mg $kg^{-1}$ and higher, and by Z-COTE and $ZnCl_2$ at 500 mg $kg^{-1}$ by 60% and 25%, respectively when compared to control.

Overall, coated Z-COTE HP1 increased root length and the concentration of more nutritional elements than uncoated Z-COTE; however, none of them affected pod production. Bulk ZnO and $ZnCl_2$ imposed more toxicity to kidney beans, since they reduced root and leaf elongation, respectively, and several essential elements in tissues.

The objectives of Stage II were to evaluate the impact of ZnO NMs in the production and nutritional quality of bean seeds cultivated in natural soil (NS) and organic matter-enriched soil (ES).

The results indicated an increase on Zn accumulation in seeds of plants grown in OM-enriched soil (38%), compared with the NS. However, in ES, exposure to Z-COTE HP1 at 125 mg $kg^{-1}$ and above resulted in lower seed Zn content, compared to bulk ZnO and $ZnCl_2$, suggesting that the hydrophobic coating interacted with soil components in a different way than uncoated ZnO. The data do not support the working hypothesis, that in the NS, Zn availability for plants exposed to Z-COTE HP1 would be lower than those exposed to Z-COTE, due to the coating's hydrophobicity. Nonetheless, it supports the hypothesis that no difference would be found in Zn availability in the ES, due to the presence of OM.

The change in OM (2.8% in the NS and 18% in the ES) content had a significant impact on the effect of the ZnO NMs or compounds in bean production and the nutrients of bean seeds. Another important soil characteristic affecting the interaction of the Zn compounds with bean plants was the pH. The reduction of pH after OM addition changed most of the minerals' mobility, by increasing their accumulation in the seeds (except Mo and Ni, which

were reduced in some cases). The increase in OM also reduced the time to reach maturity (≈25 days) and increased the seed production; however, Z-COTE HP1 and $ZnCl_2$ reduced relative sugar accumulation in the seeds, compared to seeds harvested from the NS.

As expected, there was an interaction of soil properties × compounds. For instance, plants exposed to bulk ZnO in ES were the last to reach maturity, while bulk ZnO and Z-COTE reduced the maturation time in the NS (≈12 days), compared to Z-COTE HP1 and $ZnCl_2$.

Interestingly, and in contrast to the reported toxicity of ZnO NMs, seeds from Z-COTE exposed plants only showed a significant increase in Mg (in the NS at 125 mg $kg^{-1}$) and decreases in nickel (in the ES at 125 and 250 mg $kg^{-1}$) in comparison with the controls. On the other hand, Z-COTE HP1 impacted the amounts of Mo, Ni and K, in addition to the sugar content and Zn. $ZnCl_2$ was the compound that impacted the most the nutritional content of the seeds, which could be attributed to the presence of $Cl^-$ or the high availability of Zn after dissolution. The different behavior of Z-COTE and Z-COTE HP1 opens the door for further investigations to elucidate the mechanisms of action of these compounds with different surface chemistry. Overall, the Stage II of this investigation showed that the effects of coated and uncoated ZnO NMs on bean plants are affected by the soil conditions.

The last objective of this research, Stage III, was to determine the residual effects of Z-COTE and Z-COTE HP1 into a second generation of bean seeds. Seeds obtained from plants cultivated in soil amended with either with Z-COTE, Z-COTE HP1, bulk ZnO, or $ZnCl_2$ (Stage II- S1) were grown in NMs-free soil and the produced seeds (S2)were analyzed for the nutrient composition and enzymatic activity.

Stage III provided evidence that the residual transgenerational effects of parental ZnO NMs exposure were minimal for seeds subsequently cultivated in NMs-free soil. Seed production and the time to reach maturity were unaffected. In addition, the S2 seed retained their nutritional quality, including protein, sugar, starch, and essential elements, except Ni and Ca. Nickel was reduced both in the first and second generation of seeds after exposure

to ZnO NMs, whereas the highest concentration of bulk ZnO had a residual effect that increased the accumulation of Ca in S2 seeds.

The mechanisms for these effects remain unknown, although the potential for contaminant induced genetic or epigenetic changes in the exposed parent is possible and worthy of additional investigation. While the exposure of ZnO NMs did not yield a residual negative effect in antioxidant enzyme activity, the ionic form ($ZnCl_2$) resulted in increased SOD content in S2 seeds, also possibly caused by genetic changes upon parental exposure. Further studies at the molecular and genetic level will provide the necessary insight into understanding the transgenerational residual impacts of ZnO NMs but in general, this work suggests minimal consequences of the possible incorporation of ZnO NMs into Zn-deficient soils as an agricultural amendment.

The outcomes of this investigation suggested little-to-none physiological or nutritional toxicity of ZnO NMs to bean plants and seeds under the tested concentrations and growth conditions. Regarding the different effects between Z-COTE and Z-COTE HP1, the results showed that the surface coating of Z-COTER HP1 promoted more physiological and nutritional changes both in the plant tissues and the seeds, when compared to uncoated Z-COTE. Additionally, the results suggested a transgenerational effect in the accumulation of seed nickel, caused by the exposure to Z-COTE and Z-COTE HP1. Thus, toxicity at the genetic level induced by Z-COTE and Z-COTE HP1 cannot yet be discarded, and it requires further investigation.

Additionally, the enhanced accumulation of Zn promoted by Z-COTE and Z-COTE HP1 with less negative effects than bulk ZnO or $ZnCl_2$ suggests a possible incorporation of ZnO NMs as agrochemicals. Since Zn fertilization is often required in Zn-deficient soils, this represents a viable option in agriculture. Furthermore, Zn enrichment of fruits and vegetables can fight the problem of Zn-deficiency in affected populations.

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# Curriculum Vitae

Illya Aidee Medina Velo was born on March 1, 1984 in Chihuahua, México. She earned her Bachelor of Science degree in Chemical Engineering from the Universidad Autónoma de Chihuahua in 2006. During her bachelor's studies she performed research in the Consejo Nacional de Ciencia y Tecnología (CONACYT) grant "Effect of the impregnation-drying vacuum pulse in the thermo-physical properties, quality and mass transfer of some fruits and vegetables." She completed her bachelor's thesis entitled "Impregnation of active agents in quince *(Cydonia oblonga var. Miller)* and lettuce *(Lactuca sativa var. Iceberg)* using a vacuum pulse." In 2006, she became a lecturer at the Universidad Autónoma de Chihuahua until 2008. In January of 2009 she joined the Colegio de Bachilleres del Estado de Chihuahua as a teacher, where she taught until December of 2013. While performing teaching duties, in Spring 2012, she earned her Master in Education degree from the Instituto Technológico y de Estudios Superiores de Monterrey, with her thesis: "Impact of a reading workshop in the creativity levels of high school students."

She joined the Ph.D. program in Chemistry at the University of Texas at El Paso in Spring 2014. She became a student researcher of the University of California Center for Environmental Implications of Nanotechnology (UC-CEIN), and was awarded with the CONACYT Doctoral Fellowship. Ms. Medina Velo also held an Assistant Instructor position at the Department of Chemistry during her Ph.D. studies. She received several awards, including the Graduate School Travel Award (2016), the Sustainable Nanotechnology Organization (SNO) Travel Award (2016), the First Pan American Congress of Nanotechnology Travel Award (2017), and the First CONACYT Grant Holders Meeting in North America Travel Award (2018). She has five first-author publications, and five research papers as a coauthor. She has participated in fourteen science conferences and meetings. She is currently a volunteer at SNO, where she collaborates with social outreach.

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