EFFECTS OF ZINC OXIDE NANOPARTICLES AND CARBON NANOTUBES IN IMPORTANT CROP SPECIES: PLANT GROWTH AND ELEMENT UPTAKE

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Marinus Otte
Chair

Dr. Donna Jacob

Dr. Achintya Bezbaruah

Approved:

2021-11-15
Date

Dr. Kendra Greenlee
Department Chair

ABSTRACT

Engineered nanomaterials (ENMs) have been developed for various uses in the agriculture industry. The effects of ENMs in plant systems are not yet fully understood. Some studies have reported the different effects of ENMs in plants from enhanced growth and nutrient uptake, to reduced root growth or damaged tissues. Zinc oxide nanoparticles and carbon nanotubes are commonly studied ENMs. Each have potential applications in agriculture which could benefit efficiency of farming practices or contribute to solutions for hunger and malnutrition. It is important to gain further understanding of the effects of these nanomaterials within important crop species, to facilitate their development and to support responsible use within agricultural applications. In the present study, it was found that zinc oxide nanomaterials can enhance biomass of wheat, but carbon nanotubes reduced biomass in rice. Element uptake was affected by zinc oxide in wheat and by carbon nanotubes in rice.

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DEDICATION

To Juniper

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LIST OF ABBREVIATIONS

AC .....Activated carbon

CNT.....Carbon nanotubes

ENM.....Engineered nanomaterial

IND CNT .....Industrial carbon nanotube

MWCNT .....Multi-walled carbon nanotubes

NP .....Nanoparticle

PURE CNT .....Pure carbon nanotubes

SWCNT.....Single-walled carbon nanotubes

1. INTRODUCTION

Nanoparticles (NP) exhibit unique chemical, physical, and mechanical properties. The unique properties of NPs are driven by their small size, and subsequently, the quantum size effect that occurs at the nano-scale (NNI 2019). Changes in properties at the nanoscale vary according to the type of NPs. For example, the melting point of bulk gold is 1,064 °C but can be as low as 700 °C for gold nanoparticles (Klabunde 2001), and carbon nanotubes (CNT) are highly conductive with a relatively high tensile strength compared with bulk carbon (Dalton et al. 2003).

Some nanoparticles are naturally occurring, such as those observed in volcanic ash. Nanoparticles which have been produced for a specific use are referred to as "Engineered Nanomaterials" (ENMs) (Ellenbecker and Tsai 2015). NPs have applications across many industries including computer technology, medical treatments, fertilizers, herbicides, pesticides, food processing/packaging, cosmetics, and industrial coatings. The potential applications of ENMs in the agricultural and food production industry have driven a sharp increase in research, development, and production. Some ENMs may also improve crop yields and efficiency of farming practices (Rai et al. 2018) and ENMs may also fill central roles in addressing the issue of malnutrition, which effects millions of people worldwide, via biofortification of agricultural species (De La Torre-Roche 2020).

Many studies show that ENMs can affect plant growth and nutrient uptake in various ways, and those effects are dependent on a multitude of variables. These variables may include the type of ENM, plant species, exposure method, exposure concentration, exposure duration, and/or environmental conditions. Understanding the effects of ENMs in plant systems is of particular interest due to the rapid development of these materials. Current development of

ENMs include applications such as targeted fertilizers (Dimkpa and Brindraban 2018) and pesticides (Sandhya et al. 2017 and Cao et al. 2018), higher efficiency herbicides/fungicides (Dimkpa et al. 2013; Rajiv, Rajeshwari, and Venckatesh 2013; Oliveira et al. 2015), and nano-sensors for determining real-time field conditions (Esser at al. 2012). The observed effects of ENMs in plant systems have ranged from toxicity to altered plant yields (biomass), enhanced growth, and altered nutrient uptake/partitioning (Table 1). Important crop species like wheat and rice have been shown to have varied responses upon exposure to ENMs. Because of the significance of these crops as food sources for billions of people, these crops may be recipients of treatments that utilize ENMS. The ongoing development of ENMs for uses in agriculture creates a need for further research to gain information on how ENMs effect plant systems, especially in important crop species.

Table 1**.** Effects of various ENMs in plants.

ENM	**Species**	**Concentration**	**Effect**	**Reference**
Ag	Cucumber	500 – 3000 mg kg^{-1}	Increased Ag concentration in the whole plant, improved growth indices	Shams et al. 2013
Ag	Maize	73.4 mg L^{-1}	Inhibited germination	Pokhrel and Dubey 2013
Au	*Arabidopsis thaliana*	10, 80 mg kg^{-1}	Improved seeds germination and growth, increased seed yield	Kumar et al. 2013
CeO	Soybean	0, 500, 1000, 2000, 4000 mg L^{-1}	CeO was taken up in soybeans and also caused genotoxicity	Lopez-Moreno et al. 2010
CuO	Perennial ryegrass	10-1000 mg L	DNA damage, root development stunted	Atha et al. 2014
Fe2O4	*Arabidopsis thaliana*	400 – 4000 mg L	Root length reduced	Lee et al. 2010
Mg	Corn	50-1000ug mL^{-1}	High concentration treatments improved seed germination and enhanced growth	Shinde et al. 2020

Table 1. Effects of various ENMs in plants (continued).

ENM	Species	Concentration	Effect	Reference
TiO_2	Rice	20, 30 ug mL^{-1}	Reduced Cd toxicity and enhanced growth	Rizwan et al. 2019
Ti	Wheat, Canadian waterweed (*Elodea canadensis*), curly dock (*Rumex crispus*)	0, 8, 18 mmol L^{-1}	Increase uptake of Ti	Jacob et al. 2013
Zinc complexed chitosan	Wheat	40 mg L^{-1}	Increased zinc content in grain	Dapkekar et al. 2018
ZnO NP		10 mg L^{-1}	Improved germination, increased water uptake in seed, increased photosynthetic pigments	Rai-Kala and Jajoo 2021
		40, 80, 120 mg kg^{-1}	Increased absorption of zinc and reduced leaching into surrounding soils	Sheoran 2021
	Cucumber	400 mg kg^{-1}	Increase starch content and increased Mn content, but reduced Cu content.	Zhao 2014
	Glycine max	500 mg L^{-1}	Higher Zn accumulation within plant, inhibits seed production	Zhu et al. 2019
		0.05, 0.1, 1.0 g kg^{-1}	Leaf damage at highest treatment concentration (1.0 g kg^{-1})	Preister et al. 2017
	Peanut	400, 1000, 2000 mg kg^{-1}	Increased root and stem growth as well as pod production	Prasad et al. 2012
Multi-walled Carbon Nanotube	Wheat	0-50 ug L^{-1}	Enhanced growth and increased yield	Joshi et al. 2018
	Rice	400 mg L	Yield decreased	Lin et al. 2009
	Barley, soy bean, corn	0-100 ug mL^{-1}	High concentrations enhanced germination and seedling growth	Lahiani et al. 2013
	Rice	1000, 2000 mg $L^{=1}$	Reduced root and shoot growth	Begum et al. 2014

Table 1. Effects of various ENMs in plants (continued).

ENM	Species	Concentration	Effect	Reference
Single-walled carbon nanotubes	*Arabidopsis*	12, 25, 50, 100 μg ml^{-1}	Traverse cell walls	Yuan et al. 2011
	Rice	50 μg ml^{-1}	Enhance seed germination and increase water content	Nair et al. 2012
	Maize	20 mg L^{-1}	Inhibit root hair elongation	Yan et al. 2013
Graphite nano-powder	Rice	320 mg	Application throughout plant growth enhanced growth and yield of rice.	Panigrahy et al. 2021

1.1. Zinc Oxide Nanoparticles and Carbon Nanotubes

The present study focused on carbon nanotubes (CNTs) and zinc oxide (ZnO) nanoparticles, both of which hold great potential for applications in agriculture. Both CNTs and zinc oxide NPs have received a fair amount of attention for their unique characteristics and potential applications across multiple industries. Carbon is a main component of several types of ENMs that are already being used extensively in various industries including computer hardware, sporting goods, medicine, and food processing and packaging. Zinc oxide NPs are also used extensively in industrial coatings and cosmetics.

CNTs can be single-walled or multi-walled, which refers to the number of graphene layers included. Graphene is comprised of single layer of carbon atoms arrange in a hexagonal patter. Nanotubes are formed when the graphene is rolled into a cylinder shape. Single-walled carbon nanotubes (SWCNT) consist of a single layer/sheet of graphene, while multi-walled carbon nanotubes (MWCNT) consist of two or more layers/sheets of graphene tubes. Other carbon-based nanomaterials include fullerenes, graphene, graphene oxide, nanodiamonds, quantum dots, and carbon nanotubes. CNTs exhibit unique thermal, electrical and optical properties (Josko and Oleszczuk 2014). Current applications for CNTs include energy storage

(Hu et al. 2019), environmental remediation of pollutants (Rodriguez et al. 2010, Bezbaruah 2014), computer technology (Xie et al. 2021), and medical/pharmaceutical (Panwar et al. 2019) industries. Carbon nanomaterials have also shown potential for or are already utilized in the agricultural industry as nano-fertilizers and/or nano-herbicides (Fincheira et al. 2021).

Metal-oxide ENMs also have various application in industries such as optical sensors/electronics (Djurišić, Leung 2006), cosmetics, agriculture (Sabir et al. 2014), and medical/pharmaceutical (Droepenu 2022). Zinc oxide NPs in particular have shown promising results in the agricultural industry as they have been found to enhance plant growth, yield, and nutrient uptake (Elshayb et al. 2021). Zinc oxide NPs have also been shown to have antimicrobial effects (Kranoi et al. 2021).

1.2. Zinc Oxide Nanoparticles and Wheat

Wheat is a staple food crop providing a significant proportion of the caloric intake for millions of people worldwide. It has also been identified to have potential for biofortification with zinc in order to address health problems related to zinc deficiencies that affect billions of people worldwide (Kutman et al. 2011, Shiferaw 2013). The naturally occurring zinc content of wheat grains is relatively low (Cakmak and Kutman 2018). Improvement of wheat production has largely focused on increasing yields in order to meet the higher food demands of the growing world population. However, zinc content has not increased in tandem with yield, causing a dilution effect of Zn in wheat grains (Shewry et al. 2016). Efforts are ongoing in order to find a balance between increasing wheat yields while also increasing grain zinc content (biofortification) (Xia et al. 2020)

Zinc is an essential micronutrient for humans, but it is also essential for many physiological processes within plant systems. In the agricultural industry, zinc oxide NPs have

been proposed for use in nanofertilizers. Fertilizers that utilize zinc oxide NPs have been shown to enhance plant growth (Rai-Kala and Jajoo 2021), and also can increase fertilizer efficiency by reducing the amount of zinc that leachesaway into soils (Sheoran et al. 2021).

Previous research has also shown that zinc oxide NPs can inhibit growth of wheat plants (Dimkpa et al. 2012), and that exposure to excess zinc can have a toxicity effect within plants (Tripathi et al. 2015). Authors Lin and Xing (2008) found that upon exposure to 1000 mg L^{-1} zinc oxide nanoparticles, *Lollium perenne* (ryegrass) plants exhibited reduced biomass, cell damage, and shrunken or broken root tips. In *Oryza sativa* (rice) exposed to 500 mg L^{-1} zinc oxide NPs, authors observed stunted root growth (Boonyanitipong 2011).

However, more recent studies have reported the positive effects of zinc oxide NP in wheat. Depkaker et al. (2018) reported that zinc oxide NP treatments can increase zinc content in wheat grains. Similarly, Baddar and Unrine (2018) reported that zinc oxide NPs enhanced germination, seedling growth, and zinc content in plant tissues when compared to zinc sulfate. These are promising results as developments continue for the application of zinc oxide NPs as nano-fertilizers.

Most studies on the effects of zinc oxide NPs in wheat have been limited to short-term (observing only germination and seedling growth), or field studies where external variables may be unpredictable and interfere with experiment outcomes. This research aims to investigate the long-term (complete growth cycle) effects of zinc oxide NPs in two species of wheat in a controlled greenhouse or growth chamber setting. Of particular interest are the effects of zinc oxide nanoparticles on plant growth (biomass) and element uptake (biofortification).

1.3. Carbon Nanotubes and Rice

Rice is considered a staple food source for billions of people around the world. Rice is cultivated extensively and also occurs naturally in some wetland ecosystems. Rice is also being studied for use in remediation of agricultural nutrient runoff (nitrogen and phosphorus) in waters where hyper-eutrophication is an identified issue (Moore et al. 2007,Strivastava et al. 2017). There are various methods under development for improving yields of rice (Khush 2013) and nanofertilizers may also be a part of this solution (Majeed et al. 2020).

CNTs are especially interesting in the agricultural industry due to their unique ability to interact with plant tissues (Joshi et al. 2020) and they can also be functionalized in order to enhance specific interactions within plant systems (Fincheria 2021). There have been varied responses observed in plant systems that have been exposed to CNTs. Plant responses have ranged from increasing yield/biomass (Khodakovskaya et al. 2011) to inducing DNA damage (Ghosh et al. 2011). Joshi et al. (2018) also found that application of CNTs resulted in enhanced growth in *Triticum aestivum* (wheat), and germination of castor seeds was also enhanced by application of CNTs (Fathi et al. 2017). Begum et al. (2014) observed reduced root and shoot growth in plants exposed to high concentrations of MWCNTs (1000 and 2000 mg L^{-1} hydroponic solutions). Tiwari et al. (2014) investigated the responses of maize (*Zea mays*) seedlings in growth medium with presence of carbon nanotubes and found that growth and water uptake were enhanced. CNTs have also been shown to traverse plant cell walls (Yuan et al. 2011), increase water content in germinating seedlings, enhance seed germination (Nair et al. 2012), induce plant cell death (Shen et al. 2010), inhibit root hair elongation (Yan 2013), and alter the conformation of plant DNA (Katti et al. 2015). CNTs may also indirectly impact plant growth by interfering

with availability and uptake of organic compounds in the soil (Xia et al. 2010) and Jin et al. (2014) observed altered microbial activity in soils exposed to CNTs.

Previous research into the effects of carbon nanomaterials on rice has shown mixed results. Nair et al. (2012) observed increased germination rates of rice grown in a medium spiked with different types of CNTs at a concentration of 50 $\mu g\ mL^{-1}$. CNTs have also been shown to enhance leaf growth and increase chlorophyll content in rice seedlings (Zhang et al. 2017). However, the negative effects of CNTs in rice have also been well-reported. Shen et al. (2010) demonstrated that CNTs can induce programmed cell death in rice. While treatments of CNTs at a concentration of up to 100 ug L^{-1} were shown to enhance germination and root elongation in rice, higher concentration treatments (150 ug L^{-1}) reduced root length and root activity (Jiang et al. 2014). Hao et al. (2016) also demonstrated that CNTs can cause decreased nitrogen assimilation, inhibiting plant growth in rice.

The continued development of CNTs, especially for use in agriculture (Strivastava et al. 2016), creates opportunities for their release into the environment and biological systems. Because CNTs do not readily biodegrade, they are likely to persist in the environment, especially in soils and aquatic ecosystems (Klaine et al. 2008). The potential impact of CNTs to plants varies with plant species, nanomaterial type, concentration, and route of exposure. Over time, CNTs may affect plant growth or may be transferred from plants to the food web, and eventually to humans through uptake and bio-accumulation. This becomes more likely over time as CNTs are developed since they will likely persist within the environment (soil, water, and air)

Few reports exist on the effect of carbon on uptake and metabolism of nutrients (Nowack and Bucheli 2007, Tiwari et al. 2014, Yang et al. 2013). However, Ghosh et al. (2011) found that CNTs can cause damage to DNA in plant and animal cells and Shvedova et al. (2003) observed

cell damage and morphological changes in human cells that were exposed to CNTs. Due to the increasing potential for presence of CNTs within the environment, it is important to understand the interactions that may occur when plants, especially important crop species like rice, are exposed to CNTs.

1.4. Research Aims

Both CNTs and zinc oxide NPs can have varied effects on plant growth and nutrient uptake and exposures at high concentrations of either nanoparticle type can result in toxicity effects or inhibited growth. It is important to have a better understanding of the interactions between ENMs and crops species under various growth conditions. Additionally, the development of CNTs and zinc oxide NPs for use in agricultural applications should take into consideration not only the potential benefits, but also the potential negative effects in plant systems. The general hypotheses for the current research are 1) Plants exposed to ENMs will show altered growth/biomass when compared to plants that were not exposed to ENMs; and 2) Exposure to ENMs will result in altered uptake of elements when compared to plants that were not exposed ENMs.

This research aims to investigate the potential effects of ENMs on biomass and nutrient uptake in three important crop species, *Triticum aestivum* L. subsp. *aestivum* (spring wheat), *Triticum turgidum* L. subsp. *durum* (durum wheat), and *Oryza sativa* (rice), in two greenhouse experiments. The first two experiments involved the application of zinc oxide NPs to the two species of wheat. In one experiment, zinc oxide NPs were applied to the roots of the plant, while in the parallel experiment zinc oxide NPs were applied to the plant by a foliar spray. Both soil amendments and foliar spray applications are utilized in agricultural operations. We set up two experiments (one for each type of application) in order to analyze plant responses under the

different application methods. The third experiment involved the growth of rice in nutrient solution to simulate natural growth conditions for rice. The nutrient solution was then supplemented with single walled carbon nanotubes. At the time of experimental design for this project, studies examining the effects of nanoparticles on plants were mostly limited to short term experiments examining effects on germination or the first stages of plant growth. Each of the current experiments continued well beyond the germination/seeding growth stages in order to capture the effects of ENMs in plant systems during later stages of growth.

2. ZINC OXIDE NANOPARTICLES ALTER ELEMENT UPTAKE AND ENHANCE PLANT BIOMASS ACCUMULATION WHEAT (*TRITICUM AESTVUM)*[1]

2.1. Abstract

Zinc is an essential micronutrient, and zinc oxide NPs may aid in addressing micronutrient deficiencies and improving the efficiency of agricultural farming practices. Zinc oxide NPs have potential for use in the agricultural industry as they have been found to enhance plant growth/yield and nutrient uptake. However, the effects of zinc oxide NPs in plant systems are still not fully understood, and some studies show that zinc oxide NPs can have negative effects in plants. The present study provides additional information relating to the effects of zinc oxide NPs on the growth and nutrient uptake in two species of wheat - *Triticum aestivum* L. subsp. *aestivum* (spring wheat), *Triticum turgidum* L. subsp. *durum* (durum wheat). Two experiments were conducted to test both the foliar spray and root applications of zinc oxide NPs in two wheat species. Results show plants treated with zinc oxide NPs, compared to a control with no nanoparticle (zinc sulfate), had greater biomass and altered nutrient concentrations in leaves/seeds. However, there were no significant differences in zinc concentration that could be attributed to zinc oxide NPs. It was observed that phytotoxicity was reduced at high concentration treatments of zinc oxide NPs versus high concentration treatments of zinc sulfate. These results show that zinc oxide NPs can enhance plant growth/biomass and can also influence element uptake in wheat.

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[1] This chapter was co-authored by Hannah Passolt, Marinus Otte, Donna Jacob, and Achintya Bezbaruah. Hannah Passolt had primary responsibility for conducting experiments and collecting data, and was the primary developer of the conclusions that are advanced here. Hannah Passolt also drafted and revised all versions of this chapter. Marinus Otte served as proofreader and checked the math in the statistical analysis conducted by Hannah Passolt.

2.2. Introduction

ENMs have been in development for use in agriculture and food production systems as components of fertilizers, herbicides, and pesticides (He et al. 2019). These developments have led to increased efficacy, controlled release, and targeted delivery of fertilizers, herbicides, and pesticides within and near plant systems (Srivastava et al. 2016). The sharp increase in development of ENMs in various industries produces a need for further understanding of the potential effects on and interactions with plants that are exposed to ENMs.

Wheat is one of the most widely cultivated crops worldwide and is a staple food source for billions of people. Research into optimizing growth and yield for this species is extensive, and ENMs have great potential to be used for various applications in agriculture (Table 2). Research shows that some ENMs can be taken up by the roots and translocated to other parts of the plant. Jacob et al. (2013) found that Ti ions can be taken up from the TiO_2 nanoparticle and translocated within wheat and Lopez-Moreno et al. (2010) found that CeO_2 can be taken up and accumulated by soybeans. Fertilizers containing zinc oxide NPs have been found to enhance plant growth in wheat (Sheoran et al. 2021) and can also cause increased zinc content in the grains (Dapkekar et al. 2018).

Table 2. Studies examining the effects of zinc oxide nanoparticles on various plant species.

Plant Species	Zinc Treatment	Treatment Concentration(s)	Exposure method	Effect	Reference
Wheat	ZnO NP	10mg L^{-1}	Seeds primed with ZnO NP solution	Improved germination, increase water uptake in seed, increased photosynthetic pigments	Rai-Kala and Jajoo 2021
	Zn complexed chitosan NP	40 mg L^{-1}	NP fortified fertilizer applied by aerial spraying.	Increased zinc content in grains	Depkekar 2018
Wheat	ZnO NP	40, 80, 120 mg kg^{-1}	Foliar spray applications	Increased absorption of zinc and reduced leaching into surrounding soils	Sheoran 2021
Carrot	ZnO NP solution	50-150 mg kg^{-1}	Foliar spray applications	Biomass was greater in all parts of the plant	Elizabeth et al. 2017
Cucumber	ZnO NP	400mg kg^{-1}	Solution applications to soil substrate.	Increase starch content and increased Mn content, but reduced Cu content.	Zhao 2014
Glycine max	ZnO NP	500mg L^{-1}	Soil amendment	Higher Zn accumulation within plant, inhibits seed production	Zhu et al. 2019
	ZnO NP	0.05, 0.1, 1.0 g kg^{-1}	Soil amendment	Maintained growth, leaf damage at highest treatment concentration (1.0 g kg-1)	Preister et al. 2017
Peanut	ZnO NP	400, 1000, 2000 mg kg^{-1}	Seeds primed in NP solutions prior to planting/growth in soil substrate	Increased root and stem growth as well as pod production	Prasad et al. 2012

Zinc is an essential element for many physiological processes within plants. The uptake and accumulation of zinc is regulated and affected by several factors including nutrient parent materials, nutrients/minerals within the substrate, substrate pH, presence of zinc transporters and chelators, and root activity (Gupta et al. 2016). Zinc uptake and regulation within plants can also be affected by the presence of other cations in the substrate as some cation transport channels are non-selective and may take up other divalent cations as well as (or in-lieu of) zinc (Gupta et al. 2016). Mechanisms which regulate homeostasis of essential macronutrients in plants, specifically phosphorus and nitrogen, are also interconnected with those that regulate zinc homeostasis. Cacmak et al. (2010) found that Zn fortification in the grains of wheat plants were significantly higher in plants which were grown in soils that had been treated with nitrogen fertilizer. Conversely, uptake and accumulation of zinc has been shown to decrease as inorganic phosphorus increases (Khan et al. 2014). Certain varieties of wheat under zinc deficiencies have also been shown to increase the release of phytosiderophores which facilitate zinc uptake via the plant roots (Erenoglu et al. 1996).

Many plants, especially cereal grains, are an important source of zinc for humans, and zinc deficiency affects nearly 1/3 of the global population. Fertilizers that utilize zinc oxide NPs have been shown to enhance plant growth (Depkaker et al. 2018) and can reduce the amount of zinc that leaches into soils (Sheoran et al. 2021). Depkaker et al. (2018) also found that zinc oxide NP treatments can increase zinc content in wheat grains, which could be part of the solution in addressing zinc deficiencies in humans. However, previous research has also shown that zinc oxide NPs can inhibit plant growth (Dimpka et al. 2012), and that exposure to excess zinc can have a toxicity effect within plants (Tripathi et al. 2015).

This research aimed to assess the effects of zinc oxide NPs on the element uptake and biomass of wheat plants grown in vermiculite substrate with nutrient solution. The hypotheses are as follows: 1) plants exposed to zinc oxide NPs will have greater biomass when compared to plants treated with a bulk form of zinc; 2) zinc concentrations will be higher in seeds of plants treated with zinc oxide NP solution; 3) zinc oxide NP treatments will significantly influence the uptake of other elements when compared to bulk zinc treatments; and 4) that there will be no difference in biomass or element uptake/concentrations between two species of wheat. Two experiments were conducted to test these hypotheses. For the root exposure experiment - roots of wheat plants were exposed to zinc nanoparticles by applying a zinc oxide NP solution to the substrate. For the foliar exposure experiment – foliage of wheat plants was exposed to zinc nanoparticles by spraying zinc oxide NP solution on the foliage of the plants.

2.3. Materials and Methods

Two experiments were set up as a randomized blocks with split plot design. There were twelve plots with two species per plot. Each plot contained ten plants with five replicates of each species (Table 3). Ninety seeds of *Triticum aestivum* L. subsp. *aestivum* (hard red spring wheat - NDSU 'Glenn' variety) and 90 seeds of *Triticum turgidum* L. subsp. *durum* (durum wheat - NDSU 'Divide' variety) were germinated. Nine petri dishes were prepared by lining with filter paper and saturated with RO water. Ten seeds were placed in each dish and petri dish covers were placed on each dish. The petri dishes were wrapped in tin foil to restrict light exposure. This process was repeated for each species of wheat. The seeds were cold stratified for 8 hours at ~6 °C, and 16 hours at ~25 °C each day for a total of seven days. For each experiment, sixty seeds of each species were selected for transplant based on uniformity of size. They were transplanted to vermiculite in 0.5 L cone-shaped containers. One germinated seedling was

planted approximately two centimeters below the surface of the substrate in each cone. Each block held ten plants and ten liters of nutrient solution. Half of the plants were harvested at the half-way point of the experiment for additional analysis (not reported in this study).

Table 3. Randomized block design of treatments consisting of zinc types (Nano ZnO = zinc oxide NP, ZnO = bulk zinc oxide, ZnSO = zinc sulfate), concentrations (20 and 500 mg L^{-1} for the root application experiment; 50 and 1000 mg L^{-1} for the foliar application experiment), and number of replicates.

Experiment	Concentration	Nano ZnO (#Spring/#Durum)	ZnO (#Spring/#Durum)	ZnSO (#Spring/#Durum)
Root	20 mg L^{-1}	10/10	10/10	10/10
	500 mg L^{-1}	10/10	10/10	10/10
Foliar	50 mg L^{-1}	10/10	10/10	10/10
	1000 mg L^{-1}	10/10	10/10	10/10

2.3.1. Root Exposure Experiment

Wheat was grown in a greenhouse setting using vermiculite substrate with Harmens nutrient solution (Harmens et al. 1993) modified to contain no zinc. Nutrient solution was replaced two times per week. The zinc treatment solutions were applied to the plants at four intervals throughout the duration of the experiment - on days 14, 27, 44 and 55 after transplant.

The treatment solutions were applied directly to the surface of the vermiculite in each plant container using a blunt tip syringe. Each 50 mL treatment resulted in zinc sulfate, zinc oxide, or zinc oxide NP at concentrations of 20 mg L^{-1} or 500 mg L^{-1} within each treatment block. Treatment concentrations were determined by referencing the concentrations used in similar experiments examining the effect(s) of zinc when applied to plants (Table 4). After the application of the zinc treatments, each plant vessel was rinsed with ~100mL of nutrient solution immediately after treatment applications to aid with dispersion throughout vermiculite substrate.

Table 4. Treatment concentrations referenced from similar experiments examining plant responses to zinc nanoparticles.

Treatment Concentration	Exposure method	Reference
0.1 to 1000 mg L^{-1}	Zinc NP solution applied to seeds of corn and cabbage.	(Pokhrel and Dubey 2013)
300 mg kg-1	Foliar application of zinc solution to mung bean plants.	(Thalooth, Tawfik and Mohamed 2006)
0-200 mg L^{-1}	Foliar application of zinc oxide NP solution to wheat.	(Ebrahim et al. 2005)
10-1000 mg L^{-1}	Substrate application of zinc oxide NP to ryegrass grown in nutrient solution.	(Lin and Xing 2008)
20 mg L^{-1}	Foliar application of zinc solution to wheat grown in soil.	(Gopal 2012)
500 mg L^{-1}	Substrate application of zinc oxide NP to wheat grown in sand.	(Dimpka 2013)

2.3.2. Foliar Exposure Experiment

Wheat was grown in a growth chamber with controlled settings (16 - hour light period at 20-22 °C, eight-hour dark period at 14-17 °C) and was supplied with ten liters of purified water (reverse osmosis) water every two days until the third leaf emerged. After the third leaf emerged, applications of Harmens nutrient solution, which was modified to contain no zinc, was started and changed two times per week (Harmens et al. 1993). The treatment solutions were applied to the plants at four evenly spaced intervals throughout the duration of the experiment on days 14, 27, 44 and 55 after transplant.

The zinc treatments were applied to the leaves of the plants using pressurized garden sprayers. The sprayers were pumped five times and the solution was applied evenly in a sweeping motion over each tray for ten seconds. The solution was stirred while applying the treatments to keep the solution homogenized throughout the application process. The pressure was released between each application by releasing and re-fastening the cap of the sprayer. The

trays/plants were shielded with plastic during the treatment applications to ensure that the treatment solutions were directed only at the plant foliage and did not overspray onto the substrate. This method resulted in an application of approximately 10 mL of treatment solution distributed over the shoots. Each treatment resulted in zinc sulfate, zinc oxide, or zinc oxide NP at concentrations of 50 mg L^{-1} or 1000 mg L^{-1} within each treatment block. Treatment concentrations for each experiment were determined by referencing the concentrations used in similar experiments examining the effect(s) of zinc when applied to plants (Table 4).

2.3.3. Plant Harvest

At the time of harvesting, the plants were fully mature, and the tissues were mostly dried. The plant parts were conserved as much as possible, carefully separated, and placed in labeled paper bags for drying. The plants were dried at 60 °C for a minimum of 48 hours to complete the drying process. The high concentration (500 mg L^{-1}) treatment of zinc sulfate in the root experiment was too toxic and the plants which received this treatment were not viable for harvesting. Therefore, they are not included in the data analyses.

2.3.4. Microwave Digestion and ICP Analysis

After drying, the seeds from both experiment and the leaves from the root experiment were individually weighed, and ground to a fine powder using a mortar and pestle with liquid nitrogen. The leaves of plants from the foliar exposure experiment were not analyzed because the treatments were applied directly to the leaves and washing the leaves for analysis was not possible. The samples were predigested in 5 ml concentrated HNO_3 (plant samples <250 mg were predigested in 3 ml HNO_3) for at least 16 hours. Before microwave digestions, 5 ml of ultrapure DI water was added to each predigested sample. The samples were then digested using

a CEM Mars Xpress microwave digester with Xpress 55 ml PFA venting vessels at 200 °C for 25 minutes with a 25-minute ramp to heat program.

Digested samples were analyzed via ICP-OES (Thermo iCAP 6000) at The Connecticut Agricultural Experiment Station in New Haven, CT. Continuing Calibration Verification (CCV) checks containing twenty-two elements were performed after every twelve samples. For this sample set, the following 22 elements were analyzed: Al, B, Be, Ca, Cd, Ce, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Ti, and Zn. The detection limits for each element are shown in Table 5.

Table 5. Elements analyzed via ICP and Detection Limit (DL) in mg L^{-1}.

Element	DL (mg L^{-1})	Element	DL (mg L^{-1})
Al	0.006	Mg	0.002
B	0.002	Mn	0.004
Be	0.002	Mo	0.003
Ca	0.005	Na	0.005
Cd	0.002	Ni	0.002
Ce	0.004	P	0.005
Co	0.003	Pb	0.007
Cr	0.001	S	0.005
Cu	0.279	Se	0.010
Fe	0.004	Ti	0.001
K	0.049	Zn	0.197

2.3.5. Data Analysis

Data on elements for which greater than fifty percent of the ICP output values were below the detection limit were not further included in the data analysis and will not further be discussed. Table 6 shows the elements that were or were not included in the data analysis.

Table 6. Elements analyzed for each experiment; “Y” analyzed; “-“ excluded from statistical analysis

Element	Foliar Experiment Analyzed	Root Experiment Analyzed
Al	Y	Y
B	Y	Y
Be	-	-
Ca	Y	Y
Cd	-	-
Co	-	-
Cr	-	-
Cu	-	-
Fe	Y	Y
K	Y	Y
Mg	Y	Y
Mn	Y	Y
Mo	Y	Y
Na	Y	Y
Ni	-	-
P	Y	Y
Pb	-	-
S	Y	Y
Se	-	-
Ti	Y	Y
Zn	Y	Y

Of the remaining element data sets, values that were below the specified detection limit (censored values) were assigned a value of half of their detection limit. Blanks were averaged and subtracted from each data point. Distributions for each data set were checked for normality. Statistical analysis was performed with Minitab 18. Data sets with non-normal distributions were transformed using a Box-Cox or Johnson transformation (selected by Individual Distribution Identification in Minitab). The two-way ANOVA ($p < 0.05$ significant) was used to determine if there were significant differences between zinc treatments, treatment concentrations, or if there were significant interactions between these factors. Tukey’s pairwise test was used to determine significant differences within each of the factors.

In the root exposure experiment, the high concentration treatment of zinc sulfate had a toxicity effect and so those plants were excluded from analyses.

The results for plants treated with zinc sulfate solutions are intended to be used as a general comparison to the zinc oxide treatments, and the focus of the results will be on the comparison of the nano and bulk forms of zinc oxide treatments.

2.4. Results

2.4.1. Root Application Experiment

2.4.1.1. Spring Wheat Seeds

The biomass of seeds (Figure 1) from plants treated with zinc oxide NPs was significantly greater than seeds from plants treated with zinc oxide or zinc sulfate treatments (p = 0.002) regardless of treatment concentration.

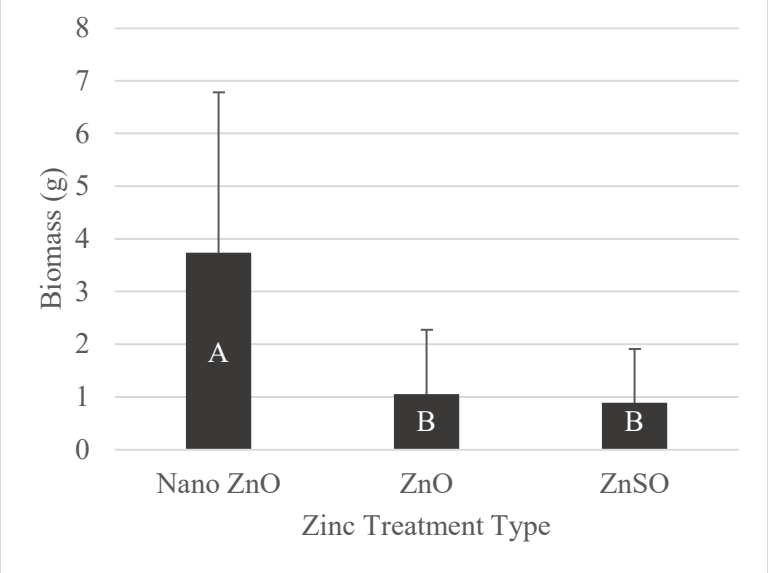

Figure 1. Average seed biomass (g) ± standard deviation in spring wheat with Nano ZnO, zinc oxide (ZnO), and zinc sulfate (ZnSO) treatments. Shared letters indicate no significant difference.

The concentrations of Zn (p = 0.023), Mn (p = 0.022), and Mg (p = 0.047) were significantly higher in seeds of plants treated with the higher concentration treatments (Figure 2).

There were no significant differences due to the type of zinc treatment nor for interactions for any of the analyzed elements in seeds of spring wheat. Summary statistics are shown in Table 7.

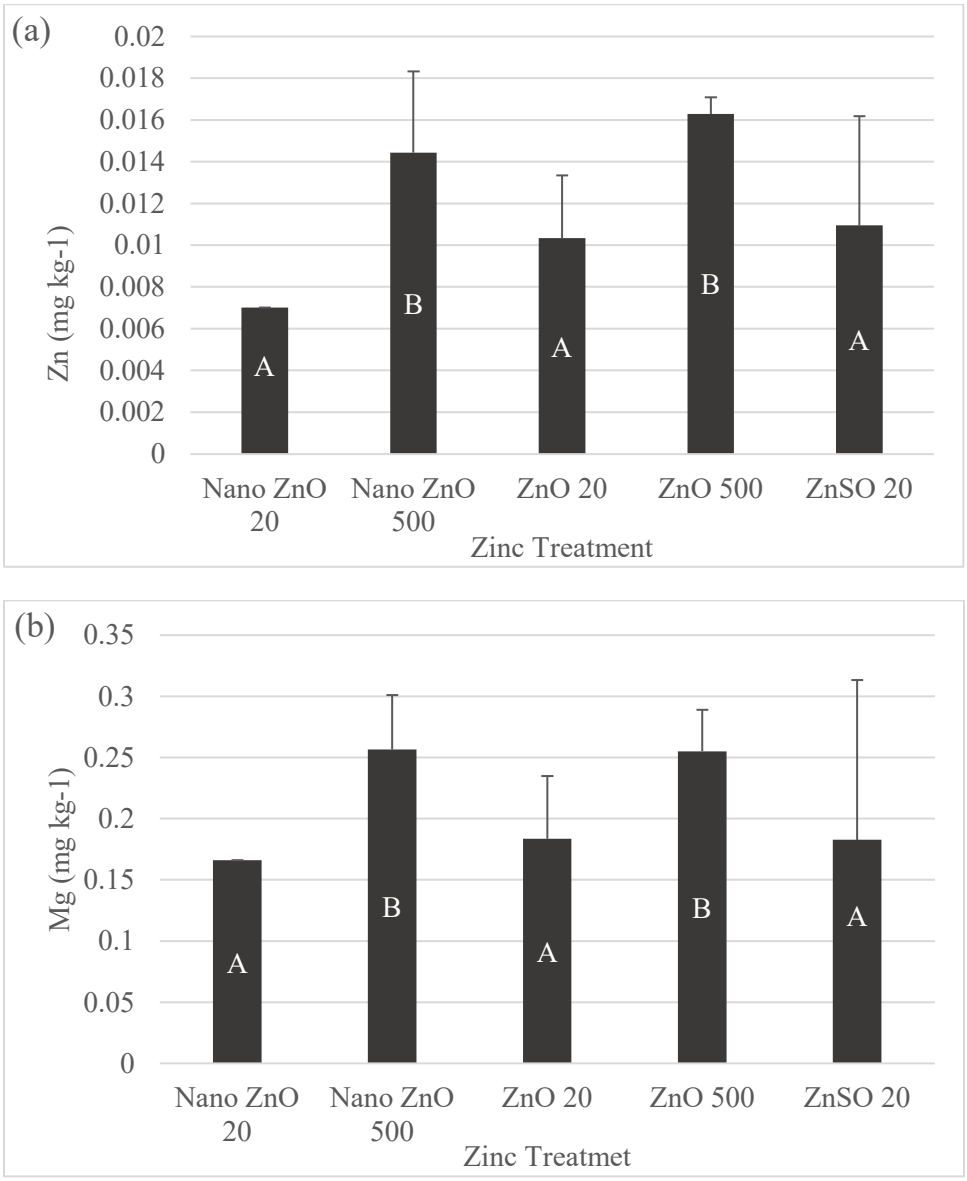

Figure 2. Average concentrations (mg kg^{-1}) of Zn (a), Mg (b), and Mn (c) concentrations ± standard deviation in seeds of spring wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments. Shared letters indicate no significant difference.

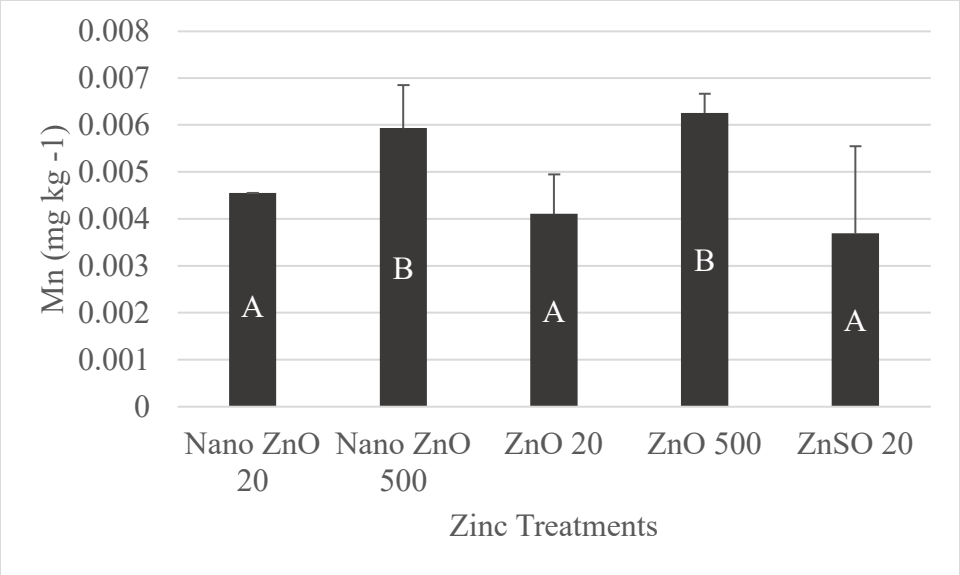

Figure 2. Average concentrations (mg kg^{-1}) of Zn (a), Mg (b), and Mn (c) ± standard deviation in seeds of spring wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments (continued). Shared letters indicate no significant difference.

Table 7. Averages (ave) and standard deviations (sd) for biomass (g) and element concentrations (mg kg^{-1}) in **seeds** of spring wheat in each treatment. Nano=zinc oxide NP, ZnO = bulk zinc oxide, ZnSO = zinc sulfate, 20 and 500 designate concentrations of zinc in nutrient solutions in mg L^{-1}. n=5. The last three columns show the results from a Two-Way ANOVA, p-values, with factors type of zinc, 'Zn-type' (Nano, ZnO, or $ZnSO_4$) concentration, 'Conc' (20 or 500 mg L^{-1}), and the interaction between the two factors, 'Interaction'. Statistically significant p-values of <0.05 are in **bold italics** for ease of reading.

Treatment	Nano 20		Nano 500		ZnO 20		ZnO 500		ZnSO 20		ANOVA analysis (*p*-values)		
	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	Zn-type	Conc	Interaction
Biomass	3.70	3.88	3.77	2.41	0.45	0.60	1.66	1.45	0.89	1.02	***0.0029***	*0.8582*	*0.5164*
Al	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.3620*	*0.7780*	*0.4970*
B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.4780*	*0.9870*	*0.4980*
Ca	0.07	0.00	0.13	0.04	0.10	0.03	0.14	0.02	0.06	0.05	*0.5210*	*0.0520*	*0.7930*
Fe	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.01	*0.1720*	*0.7560*	*0.7330*
K	1.75	0.00	1.29	0.16	1.37	0.39	1.20	0.21	0.76	0.72	*0.2500*	*0.1420*	*0.4700*
Mg	0.17	0.00	0.26	0.05	0.18	0.06	0.26	0.03	0.18	0.16	*0.8230*	***0.0470***	*0.7900*
Mn	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	*0.9230*	***0.0220***	*0.5520*
Mo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.5480*	*0.0820*	*0.6330*
Na	0.01	0.00	0.03	0.03	0.02	0.01	0.01	0.00	0.02	0.02	*0.7710*	*0.6210*	*0.3450*
P	0.72	0.00	0.86	0.08	0.76	0.22	0.86	0.09	0.58	0.41	*0.8650*	*0.2930*	*0.8800*
S	0.26	0.00	0.28	0.02	0.29	0.10	0.28	0.02	0.18	0.11	*0.7740*	*0.8530*	*0.7660*
Ti	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.2000*	*0.6270*	*0.2840*
Zn	0.01	0.00	0.01	0.00	0.01	0.00	0.02	0.00	0.01	0.01	*0.3100*	***0.0230***	*0.7650*

2.4.1.2. Spring Wheat Leaves

Plants treated with zinc oxide NP had greater biomass ($p = 0.029$) regardless of high or low treatment concentrations (Figure 3).

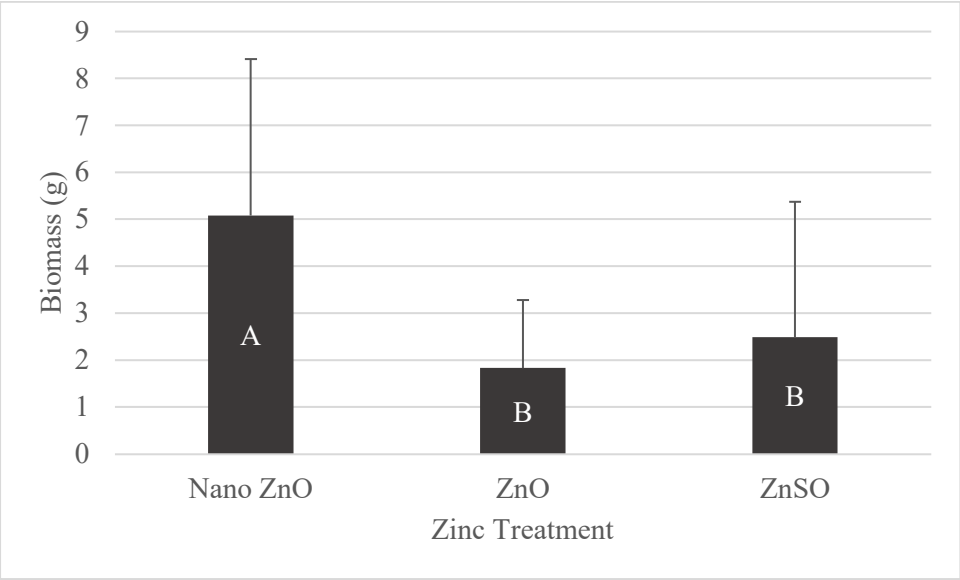

Figure 3. Average leaf biomass (g) ± standard deviation in spring wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments. Shared letters indicate no significant difference.

Results for element concentrations in the leaves showed significant differences in some elements for zinc treatment type, treatment concentration, or the interaction of these two factors (Table 8). Zinc concentrations were significantly higher in plants exposed to the higher concentration zinc treatments (500 mg L^{-1}) (Figure 4). Boron ($p = 0.045$), calcium ($p = 0.004$), and magnesium ($p = 0.003$) were all present in lower concentrations in the leaves of plants treated with zinc oxide NP when compared with zinc oxide (Figure 5).

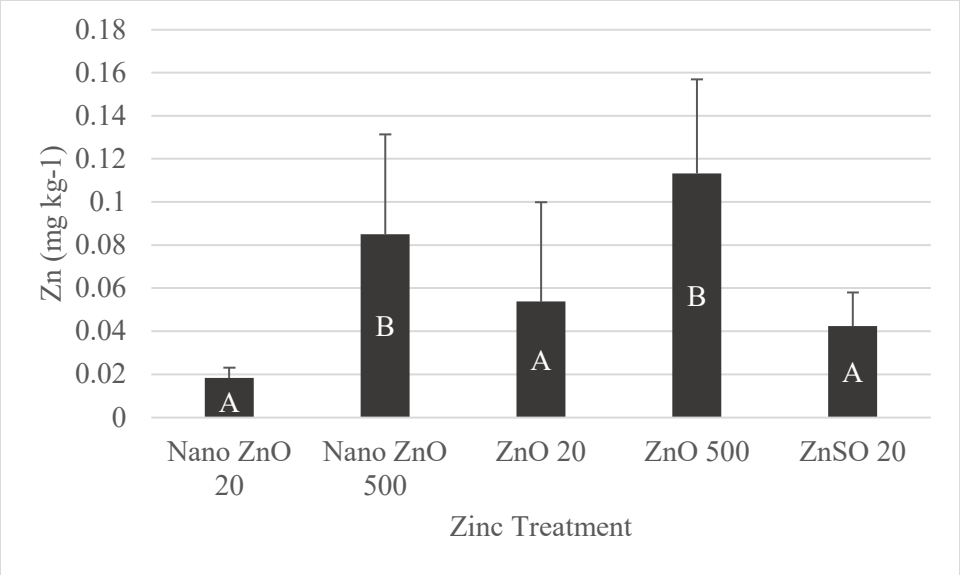

Figure 4. Average concentrations (mg kg^{-1}) of Zn ± standard deviation in leaves of spring wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1}treatments. Shared letters indicate no significant difference.

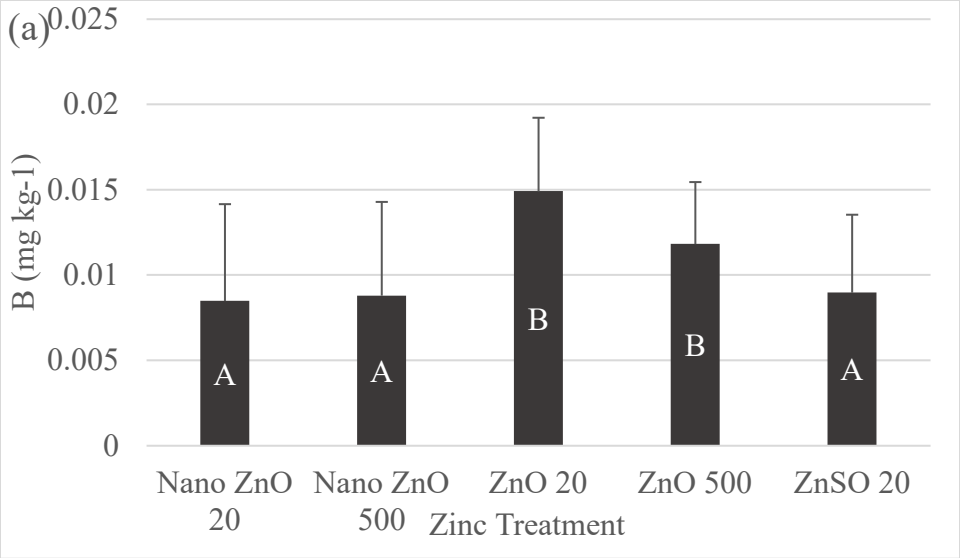

Figure 5. Average concentrations (mg kg^{-1}) of B (a), Ca (b), and Mg (c) ± standard deviation in leaves of spring wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments. Shared letters indicate no significant difference.

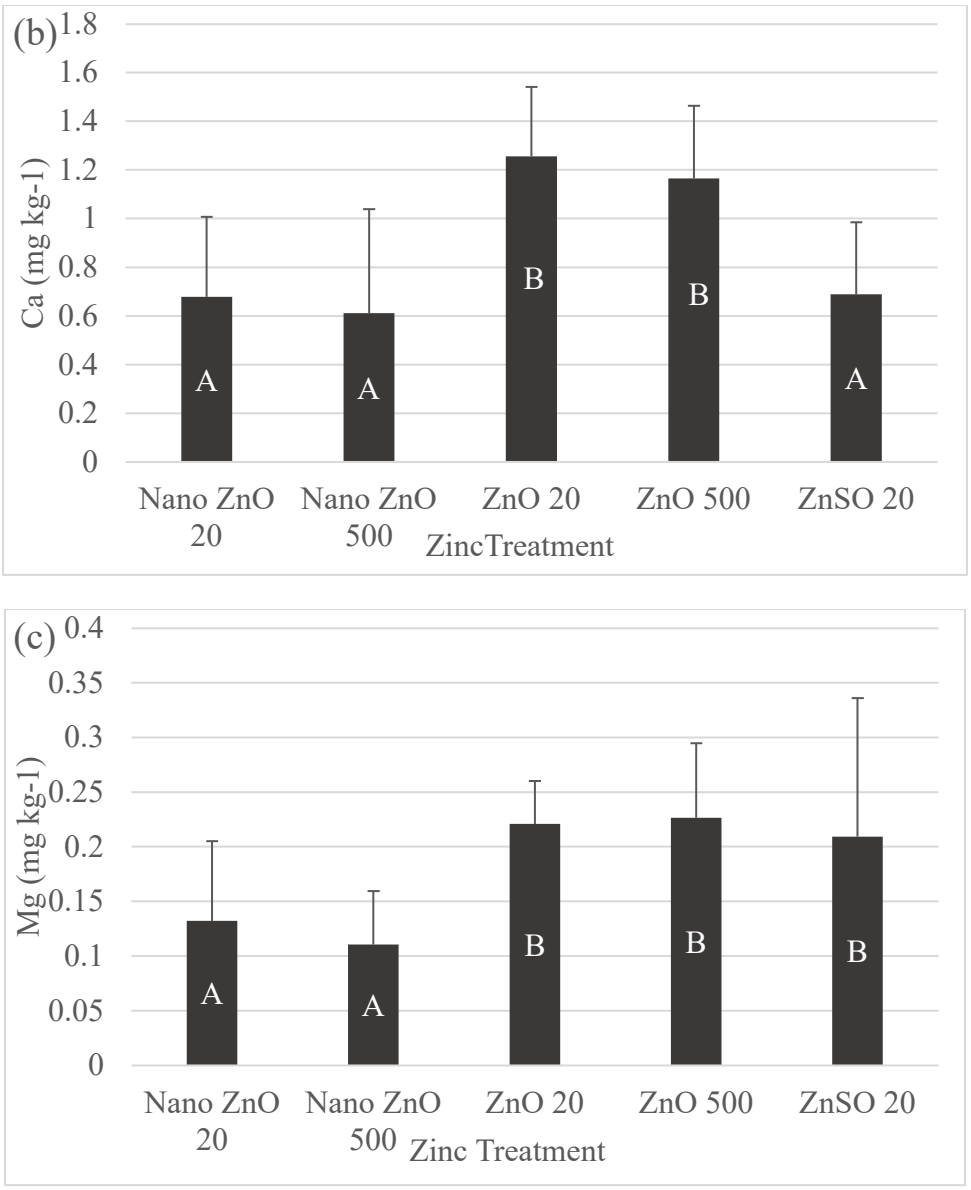

Figure 5. Average concentrations (mg kg^{-1}) of B (a), Ca (b), and Mg (c) ± standard deviation in leaves spring wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments (continued). Shared letters indicate no significant difference.

Table 8. Averages (ave) and standard deviations (sd) for element concentrations (mg kg^{-1}) in **leaves** of spring wheat in each treatment. Nano=nanoparticle zinc oxide, ZnO = bulk zinc oxide, ZnSO = zinc sulfate, 20 and 500 designate concentrations of carbon in nutrient solutions in mg L^{-1}. n=5. The last three columns show the results from a Two-Way ANOVA, P-values, with factors type of zinc, 'Zn-type' (Nano, ZnO, or $ZnSO_4$) concentration, 'Conc' (20 or 500), and the interaction between the two factors, 'Interaction'. Statistically significant P-values of <0.05 are in ***bold italics*** for ease of reading.

Treatment	Nano Zn 20		Nano Zn 500		ZnO 20		ZnO 500		ZnSO 20		ANOVA analysis (*p*-values)		
	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	Zn-type	Conc	Interaction
Biomass	4.49	5.23	5.74	4.96	17.14	20.76	15.50	11.21	4.42	4.06	***0.029***	*0.538*	*0.964*
Al	0.00	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.00	*0.429*	*0.437*	*0.641*
B	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.00	***0.045***	*0.531*	*0.444*
Ca	0.68	0.37	0.61	0.48	1.26	0.32	1.17	0.33	0.69	0.33	***0.004***	*0.650*	*0.946*
Fe	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.01	0.01	0.00	*0.069*	*0.590*	*0.811*
K	4.75	2.39	2.49	1.72	4.90	0.57	5.45	0.81	4.73	2.44	*0.116*	*0.287*	***0.034***
Mg	0.13	0.08	0.11	0.05	0.22	0.04	0.23	0.08	0.21	0.14	***0.003***	*0.793*	*0.651*
Mn	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	*0.232*	*0.904*	*0.104*
Mo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.094*	*0.458*	*0.863*
Na	0.90	0.53	0.59	0.47	0.77	0.35	0.56	0.28	0.53	0.41	*0.688*	*0.185*	*0.775*
P	0.69	0.36	0.42	0.27	0.76	0.10	0.83	0.07	0.62	0.21	*0.057*	*0.615*	*0.098*
S	0.50	0.21	0.39	0.25	0.61	0.08	0.54	0.07	0.55	0.17	*0.307*	*0.296*	*0.731*
Ti	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.503*	*0.774*	*0.594*
Zn	0.02	0.01	0.09	0.05	0.05	0.05	0.11	0.05	0.04	0.02	*0.090*	***0.003***	*0.570*

Ca (p = 0.031) and Mo (p = 0.017) were present in significantly higher concentrations in leaves of plants that were treated with low concentrations of zinc oxide versus the leaves from plants treated with low concentrations of zinc oxide NP and zinc sulfate (Table 9, Figure 6).

Table 9**.** Statistical significance (two-way ANOVA, p < .05 significant) of element concentrations in leaves treated with 20 mg L^{-1} zinc treatments (p-values within element rows indicate Tukey's pair-wise results, df = 19).

Treatment @ 20 mg L^{-1}			
Element	***p*-values**	**Element**	***p*-values**
Al	*0.389*	Mo	*0.017*
B	*0.109*	Na	*0.420*
Ca	*0.031*	P	*0.553*
Fe	*0.425*	S	*0.630*
K	*0.989*	Ti	*0.196*
Mg	*0.331*	Zn	*0.112*
Mn	*0.933*		

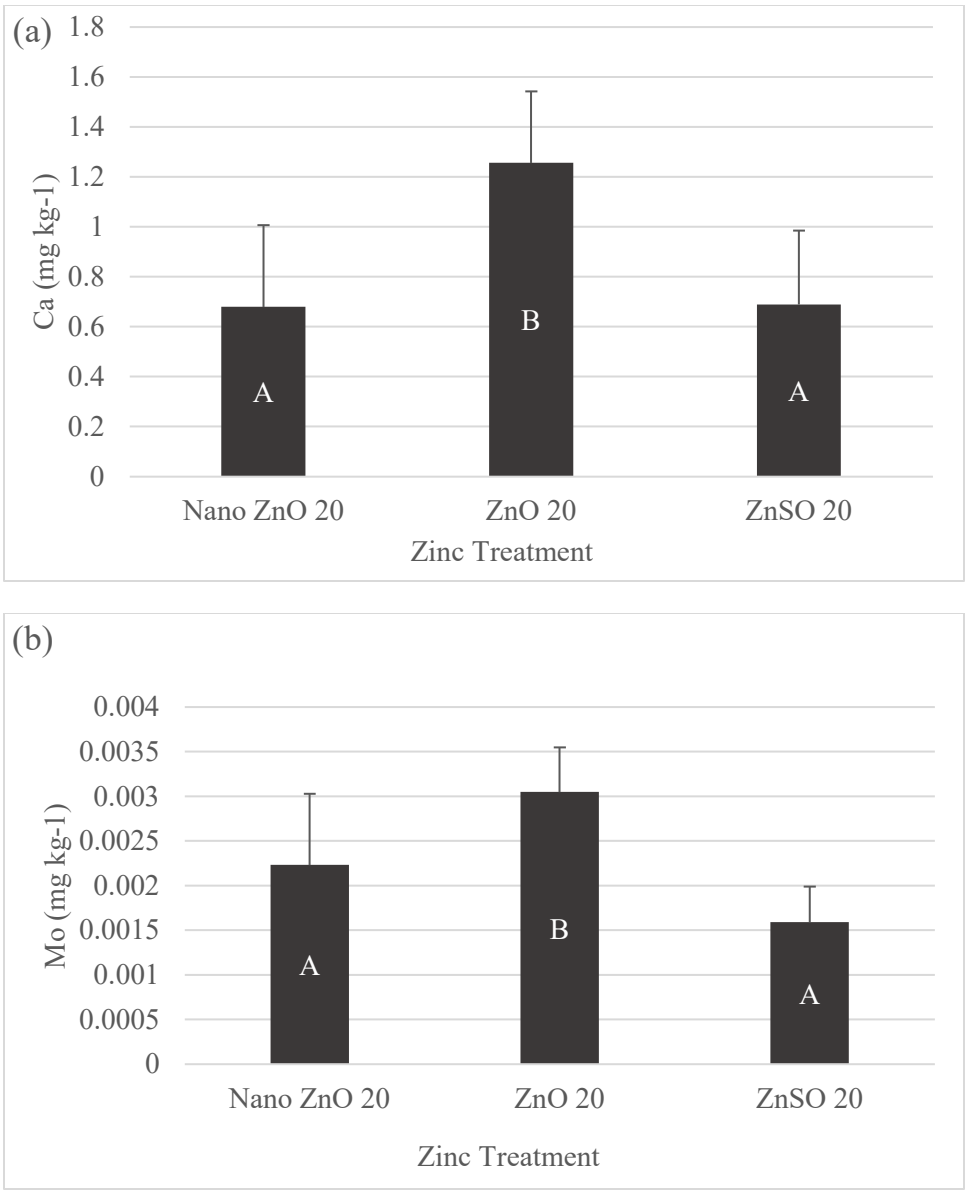

Figure 6**.** Average concentrations (mg kg^{-1}) of Ca (a) and Mo (b) ± standard deviation in leaves of spring wheat with 20 mg L^{-1} (Nano ZnO, ZnO, and $ZnSO_4$ treatments). Shared letters indicate no significant difference.

2.4.1.3. Durum Wheat Seeds

Plants treated with zinc oxide NP had significantly greater seed biomass (p = 0.003) when compared to zinc oxide or zinc sulfate (Figure 7).

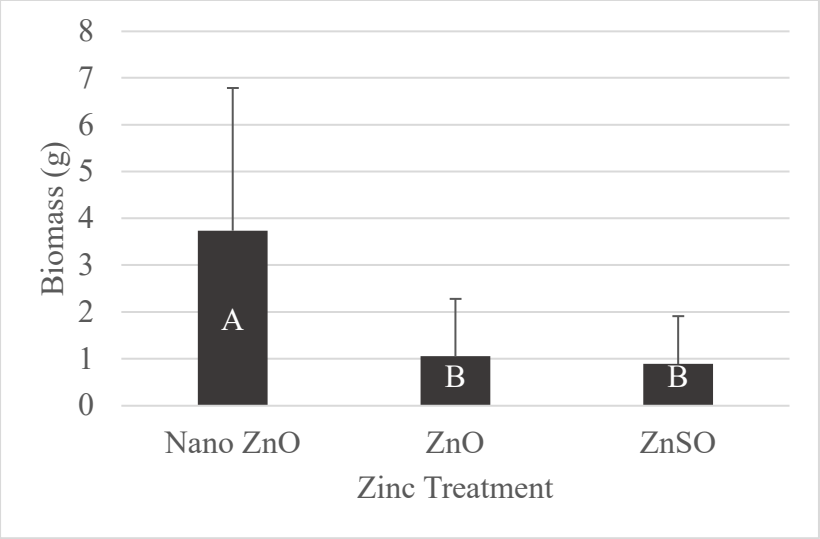

Figure 7. Average seed biomass (g) ± standard deviation in durum wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments. Shared letters indicate no significant difference.

The interaction between zinc treatment type and concentration was significant for potassium (Table 10). Potassium was present in higher concentrations ($p = 0.001$) in seeds of plants treated with ZnO nanoparticles and there was also an interaction ($p = 0.003$) between treatment type and treatment concentration. Similarly, manganese shows an interaction where the effect of concentration is reversed between treatment types and concentrations ($p = 0.005$) (Figure 8).

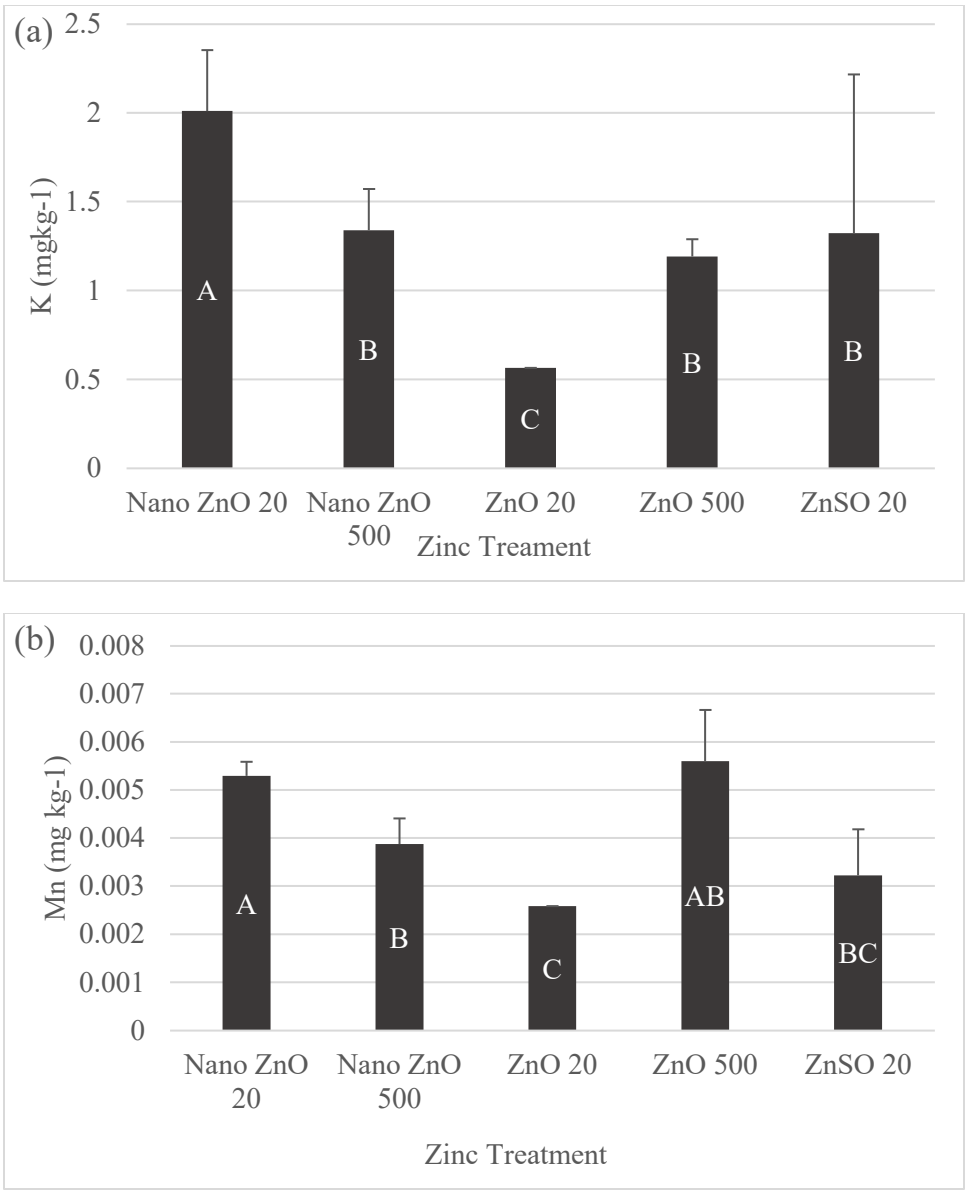

Figure 8. Average concentrations (mg kg^{-1}) of K (a) and Mn (b) ± standard deviation in seeds of durum wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments. Shared letters indicate no significant difference.

The concentration of molybdenum (p = 0.013) in seeds was significantly higher in plants exposed to treatments with higher concentrations of zinc, whether the bulk or nano form. Sulfur (p = 0.014) had lower concentrations within seeds exposed to low concentrations of zinc oxide versus nano zinc oxide or zinc sulfate (Figure 9).

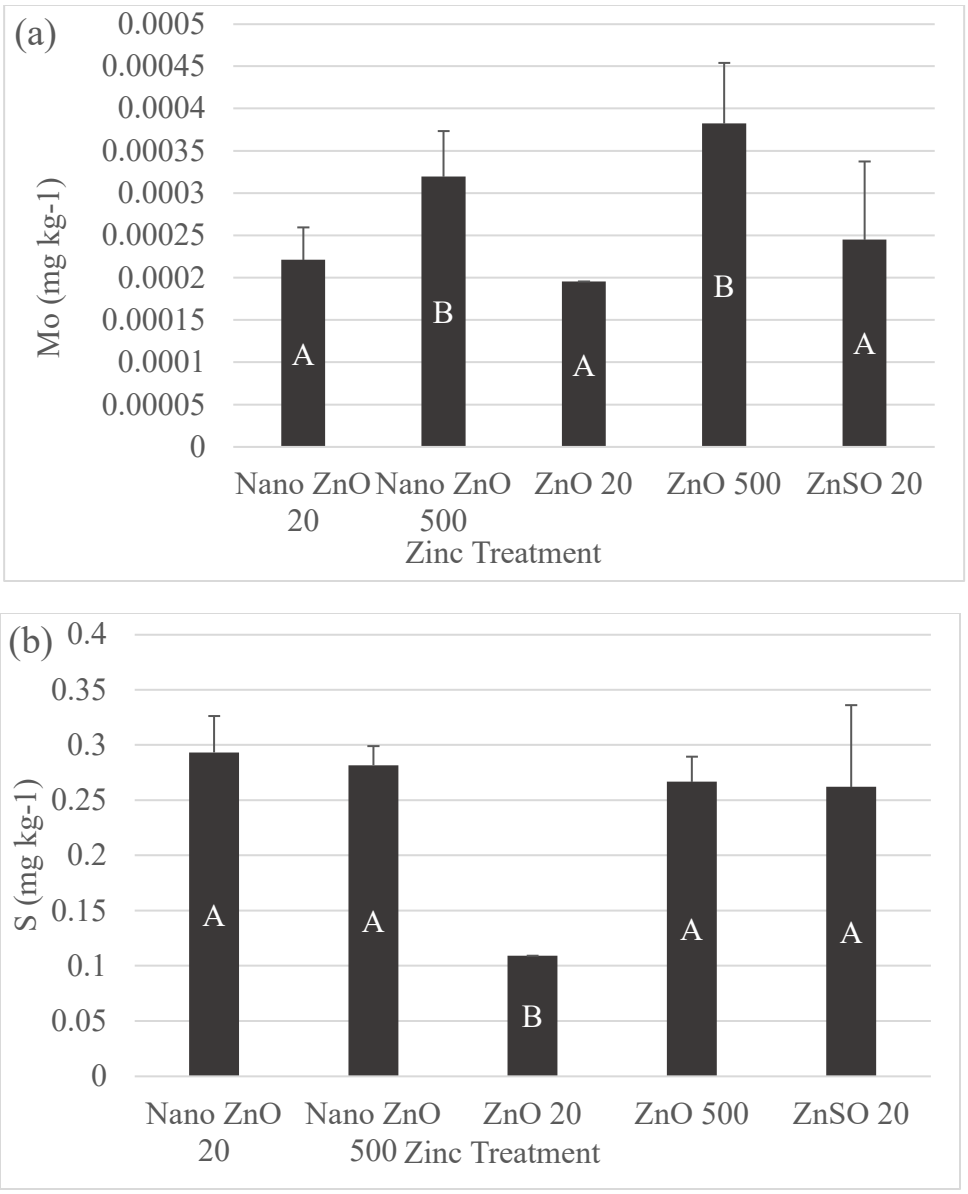

Figure 9. Average concentrations (mg kg^{-1}) of Mo (a) and S (c) ± standard deviation in seeds of durum wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments. Shared letters indicate no significant difference.

Table 10. Averages (ave) and standard deviations (sd) for element concentrations (mg kg^{-1}) in **seeds** of durum wheat in each treatment. Nano=nanoparticle zinc oxide, ZnO = bulk zinc oxide, ZnSO = zinc sulfate, 20 and 500 designate concentrations of zinc in nutrient solutions in mg L^{-1}. n=5. The last three columns show the results from a Two-Way ANOVA, P-values, with factors type of zinc, 'Zn-type' (Nano, ZnO, or $ZnSO_4$) concentration, 'Conc' (20 or 500), and the interaction between the two factors, 'Interaction'. Statistically significant P-values of <0.05 are in ***bold italics*** for ease of reading.

Treatment	Nano Zn 20		Nano Zn 500		ZnO 20		ZnO 500		ZnSO 20		ANOVA analysis (*p*-values)		
	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	Zn-type	Conc.	Interaction
Biomass	3.70	3.88	3.77	2.41	0.45	0.60	1.66	1.45	0.88	1.02	***0.0028***	*0.8582*	*0.5161*
Al	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.4460*	*0.8930*	*0.8280*
B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.9870*	*0.5630*	*0.6180*
Ca	0.11	0.04	0.08	0.03	0.08	0.00	0.13	0.01	0.10	0.02	*0.5880*	*0.4970*	*0.0670*
Fe	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00	*0.4910*	*0.9700*	***0.0330***
K	2.01	0.34	1.34	0.23	0.56	0.00	1.19	0.10	1.32	0.89	***0.0010***	*0.8850*	***0.0030***
Mg	0.18	0.02	0.16	0.05	0.17	0.00	0.25	0.03	0.17	0.04	*0.2290*	*0.3370*	*0.1090*
Mn	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	*0.4070*	*0.1980*	***0.0050***
Mo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.6770*	***0.0130***	*0.3380*
Na	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.03	0.02	*0.4240*	*0.9280*	*0.9000*
P	0.81	0.04	0.76	0.10	0.59	0.00	0.83	0.09	0.69	0.25	*0.2580*	*0.1610*	*0.0540*
S	0.29	0.03	0.28	0.02	0.11	0.00	0.27	0.02	0.26	0.07	***0.0410***	*0.4120*	*0.1580*
Ti	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.5290*	*0.5880*	*0.9850*
Zn	0.01	0.00	0.01	0.00	0.01	0.00	0.02	0.01	0.02	0.00	*0.2680*	*0.3840*	*0.3170*

2.4.1.4. Durum Wheat Leaves

Biomass of leaves treated with zinc oxide NP treatments was significantly greater than those treated with bulk zinc oxide ($p = 0.006$, Figure 10).

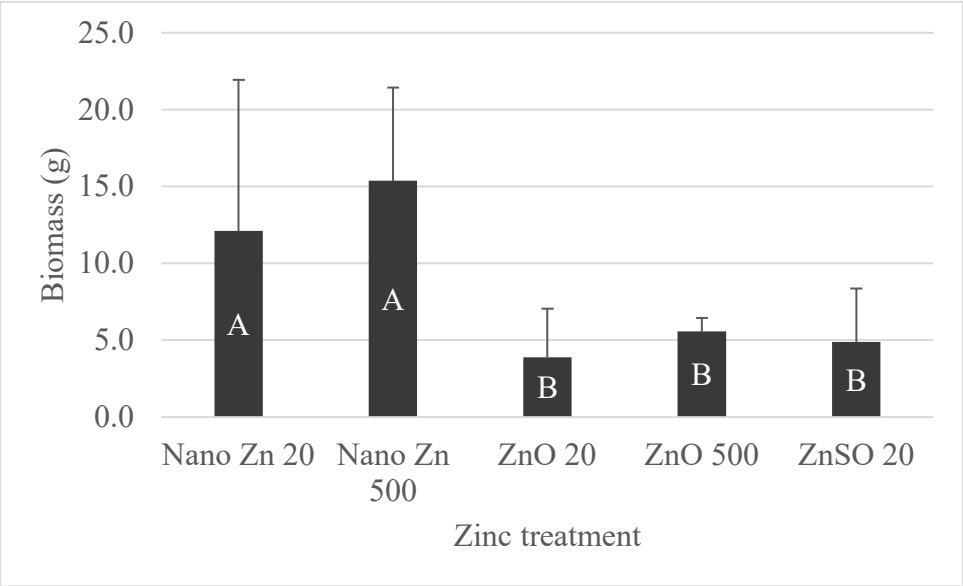

Figure 10. Average leaf biomass (g) ± standard deviation in durum wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments. Shared letters indicate no significant difference.

There were no significant differences in element uptake in the in the plants treated with the 20 mg L^{-1} zinc treatments (Table 11). However, the concentration of molybdenum ($p = 0.022$) was increased in plants exposed to higher concentrations of the zinc oxide NPs and bulk zinc oxide. Boron ($p = 0.046$) and sodium ($p = 0.036$) concentrations in leaves are higher in plants treated with zinc oxide NP compared to plants treated with zinc oxide (Figure 11). Leaves of plants exposed to high concentrations of zinc oxide also had higher concentrations of phosphorus ($p = 0.019$). Simultaneously, concentrations of phosphorus were lower when plants were exposed to high concentrations of nano-zinc treatments (Figure 12).

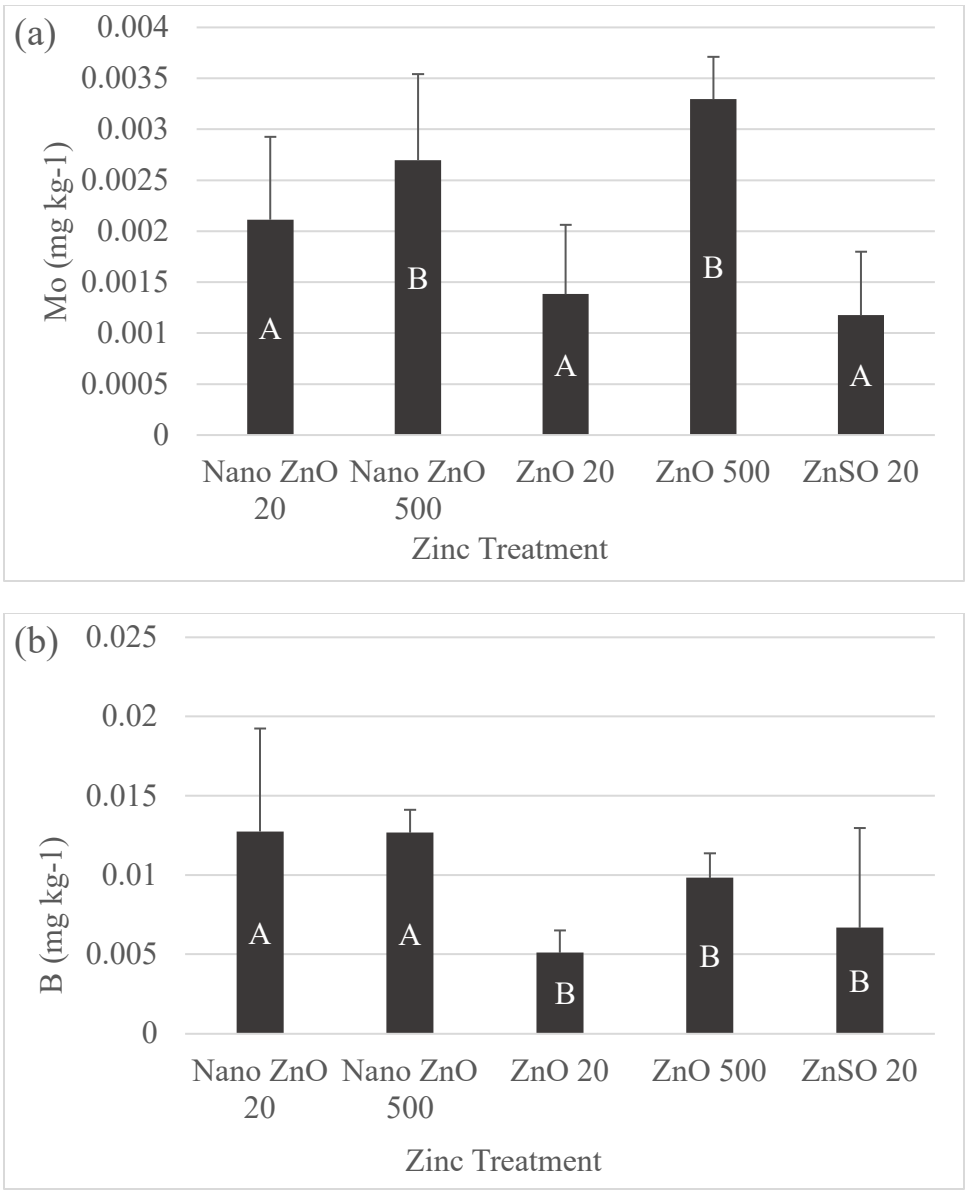

Figure 11. Average concentrations (mg kg^{-1}) of Mo (a), B (b), Na (c), and P (d) ± standard deviation in leaves of durum wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments. Shared letters indicate no significant difference.

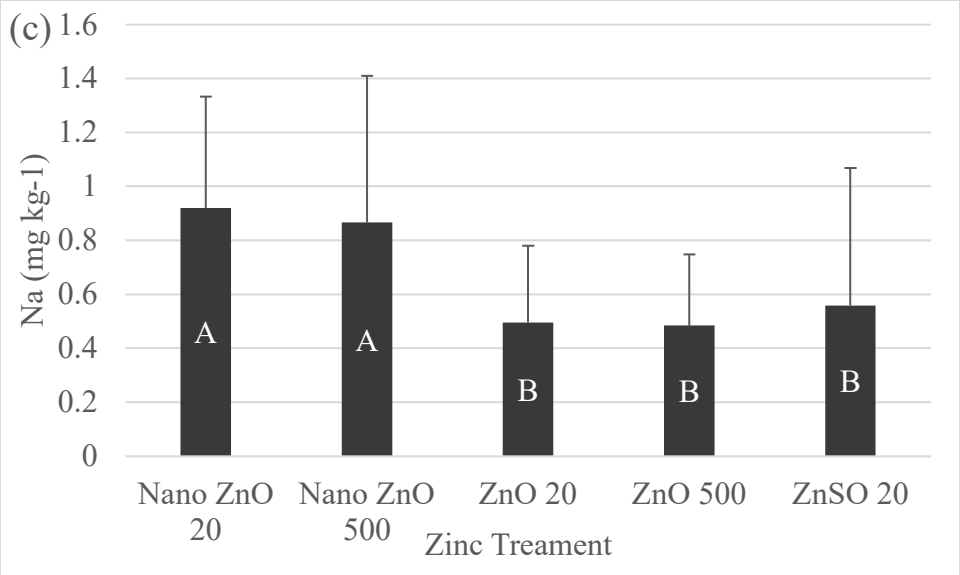

Figure 11. Average concentrations (mg kg^{-1}) of Mo (a), B (b), Na (c), and P (d) ± standard deviation in leaves of durum wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments (continued). Shared letters indicate no significant difference.

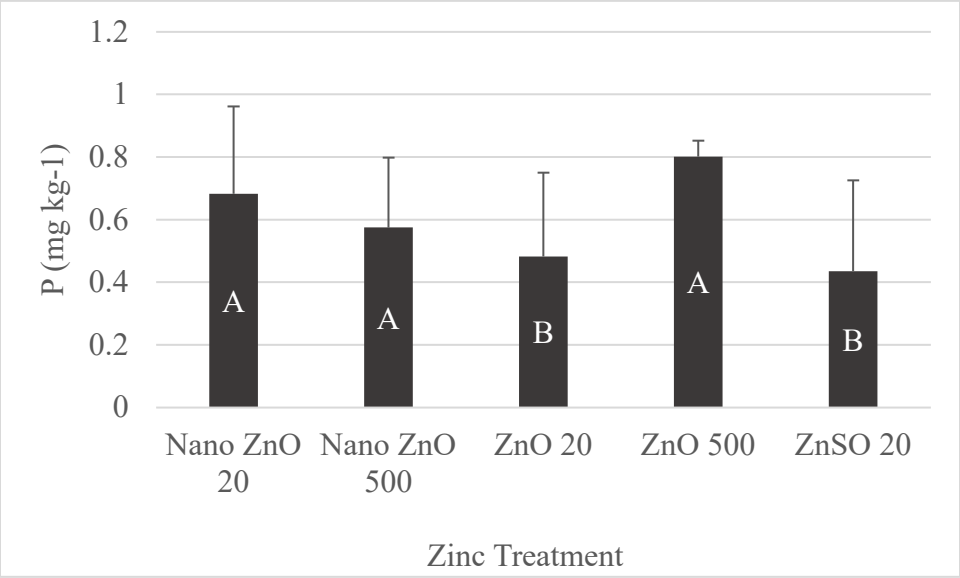

Figure 12**.** Average concentration (mg kg^{-1}) of P ± standard deviation in seeds of durum wheat with Nano ZnO 20 mg L^{-1}, Nano ZnO 500 mg L^{-1}, ZnO 20 mg L^{-1}, ZnO 500 mg L^{-1}, and $ZnSO_4$ 20 mg L^{-1} treatments. Shared letters indicate no significant difference.

Table 11. Averages (ave) and standard deviations (sd) for element concentrations (mg kg^{-1}) in leaves of durum wheat in each treatment. Nano=nanoparticle zinc oxide, ZnO = bulk zinc oxide, ZnSO = zinc sulfate, 20 and 500 designate concentrations of carbon in nutrient solutions in mg L^{-1}. n=5. The last three columns show the results from a Two-Way ANOVA, P-values, with factors type of zinc, 'Zn-type' (Nano, ZnO, or $ZnSO_4$) concentration, 'Conc' (20 or 500), and the interaction between the two factors, 'Interaction'. Statistically significant P-values of <0.05 are in bold italics for ease of reading.

Treatment	Nano 20		Nano 500		ZnO 20		ZnO 500		ZnSO 20		ANOVA analysis (*p*-values)		
	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	Zn-type	Conc	Interaction
Biomass	12.10	9.84	15.37	6.07	3.88	3.18	5.58	0.87	4.87	3.50	***0.0069***	*0.3788*	*0.7599*
Al	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.01	*0.0940*	*0.3280*	*0.2470*
B	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.01	***0.0460***	*0.4650*	*0.4460*
Ca	0.82	0.38	0.87	0.40	0.37	0.22	1.00	0.11	0.48	0.45	*0.3110*	*0.0720*	*0.2940*
Fe	0.01	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.00	*0.6100*	*0.7230*	*0.3860*
K	4.41	2.22	4.00	1.71	3.45	2.68	5.76	1.45	3.08	2.52	*0.4960*	*0.2760*	*0.1370*
Mg	0.14	0.05	0.14	0.08	0.09	0.02	0.22	0.07	0.11	0.06	*0.5560*	*0.0800*	*0.1130*
Mn	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	*0.8290*	*0.5390*	*0.1280*
Mo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.8480*	***0.0220***	*0.3070*
Na	0.92	0.41	0.87	0.54	0.50	0.28	0.48	0.26	0.56	0.51	***0.0360***	*0.8530*	*0.9130*
P	0.68	0.28	0.57	0.22	0.48	0.27	0.80	0.05	0.44	0.29	*0.6290*	*0.7900*	***0.0190***
S	0.49	0.17	0.45	0.18	0.39	0.19	0.50	0.05	0.38	0.20	*0.4970*	*0.9850*	*0.4510*
Ti	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.5630*	*0.6040*	*0.6980*
Zn	0.03	0.02	0.12	0.08	0.13	0.25	0.14	0.12	0.05	0.03	*0.4330*	*0.6200*	*0.2070*

2.4.2. Foliar Application Results

2.4.2.1. Spring Wheat Seeds

There were no significant differences (Treatment, $p = 0.124$; Concentration $p = 0.713$; Interaction, $p = 0.116$) for biomass of spring wheat seeds (Figure 13). Results for element concentrations are shown in Table 12.

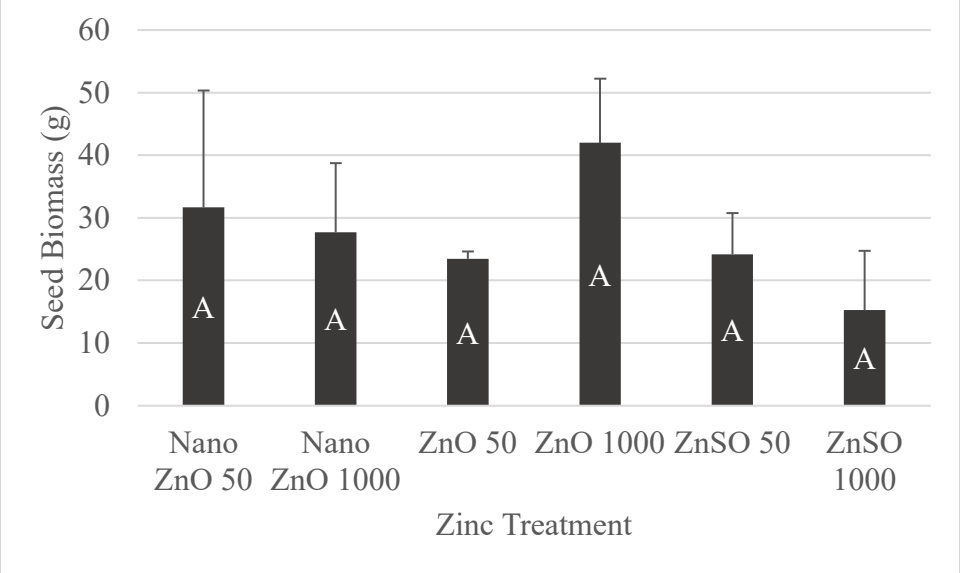

Figure 13. Average seed biomass ± standard deviation in seeds of spring wheat with Nano ZnO 50 mg L^{-1}, Nano ZnO 1000 mg L^{-1}, ZnO 50 mg L^{-1}, ZnO 1000 mg L^{-1}, $ZnSO_4$ 50 mg L^{-1}, and $ZnSO_4$ 1000 mg L^{-1} treatments.

Spring wheat seeds showed decreased calcium ($p = 0.039$) and iron ($p = 0.010$) concentrations when plants were exposed to high concentrations of zinc sulfate (Figure 14). Concentrations of calcium and iron were not significantly different between any of the other treatment groups.

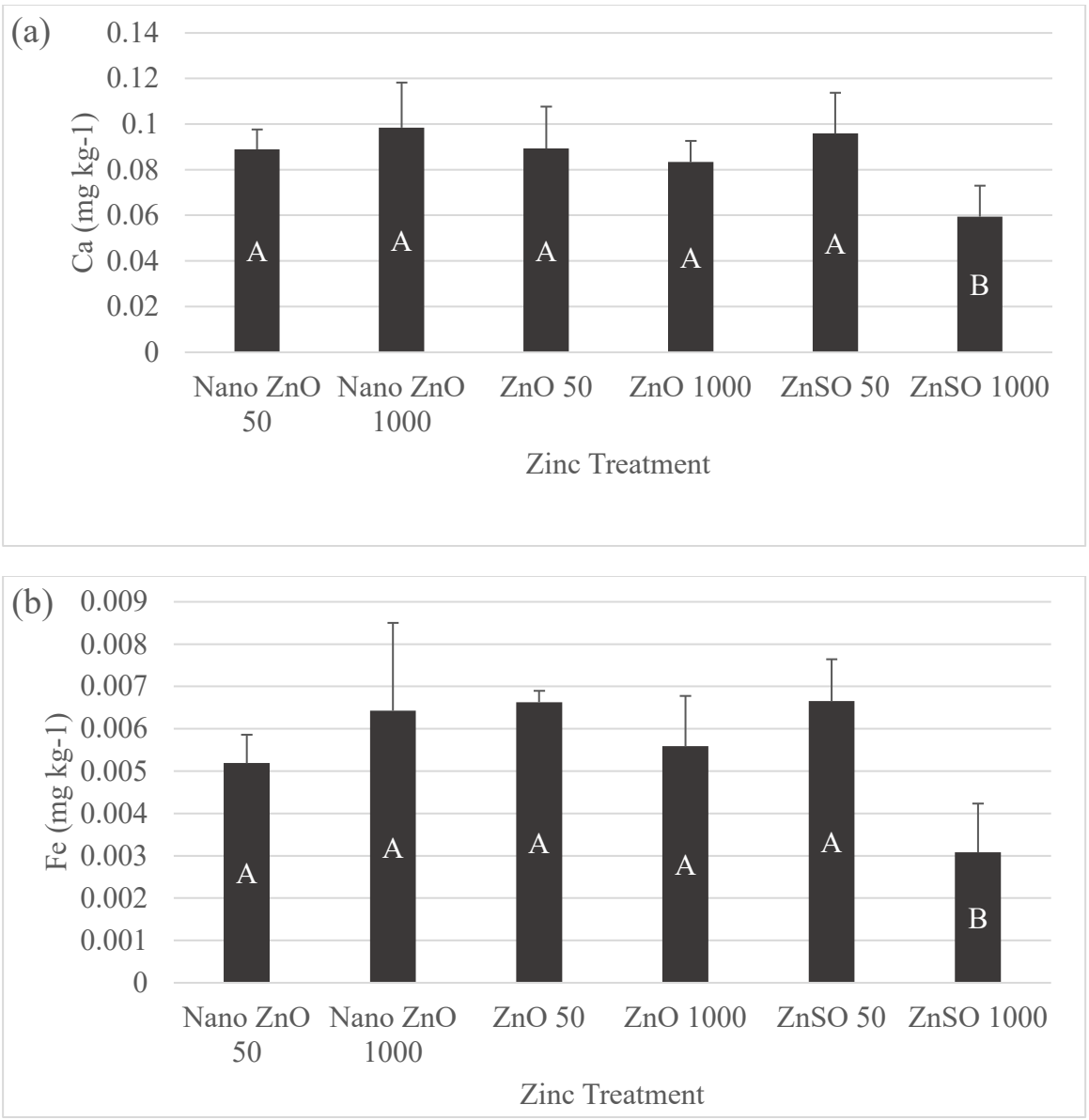

Figure 14**.** Average concentrations (mg kg^{-1}) of Ca (a) and Fe (b) concentrations ± standard deviation in seeds of spring wheat with Nano ZnO 50 mg L^{-1}, Nano ZnO 1000 mg L^{-1}, ZnO 50 mg L^{-1}, ZnO 1000 mg L^{-1}, $ZnSO_4$ 50 mg L^{-1}, and $ZnSO_4$ 1000 mg L^{-1} treatments. Shared letters indicate no significant difference.

Potassium concentration in seeds is higher (p = 0.008) when the plants are exposed to zinc sulfate as opposed to zinc oxide NP or bulk zinc oxide. Magnesium (p = 0.049) concentrations were lower in seeds exposed to higher concentrations of non-nano zinc treatments. Manganese concentrations in seeds were lower in plants exposed to zinc sulfate

versus those exposed to nano zinc oxide or bulk zinc oxide ($p = 0.006$). Sodium was present in lower ($p = 0.028$) concentrations in seeds of plants exposed to bulk zinc oxide, but concentrations increased in plants exposed to nano zinc oxide and was highest in plants exposed to zinc sulfate (Figure 15).

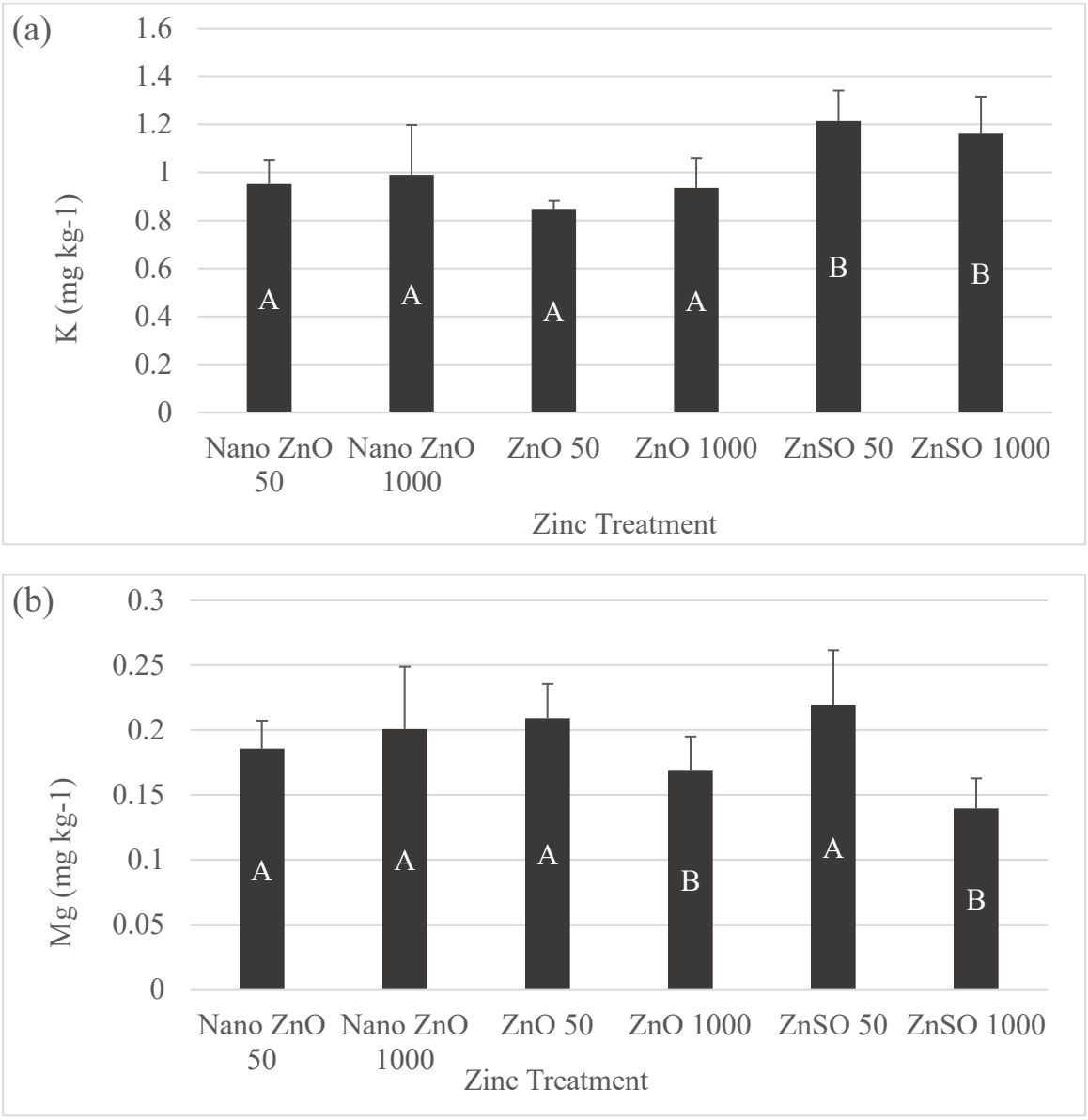

Figure 15. Average concentrations (mg kg^{-1}) of K (a), Mg (b), Mn (c), and Na (d) ± standard deviation in seeds of spring wheat with Nano ZnO 50 mg L^{-1}, Nano ZnO 1000 mg L^{-1}, ZnO 50 mg L^{-1}, ZnO 1000 mg L^{-1}, $ZnSO_4$ 50 mg L^{-1}, and $ZnSO_4$ 1000 mg L^{-1} treatments. Shared letters indicate no significant difference.

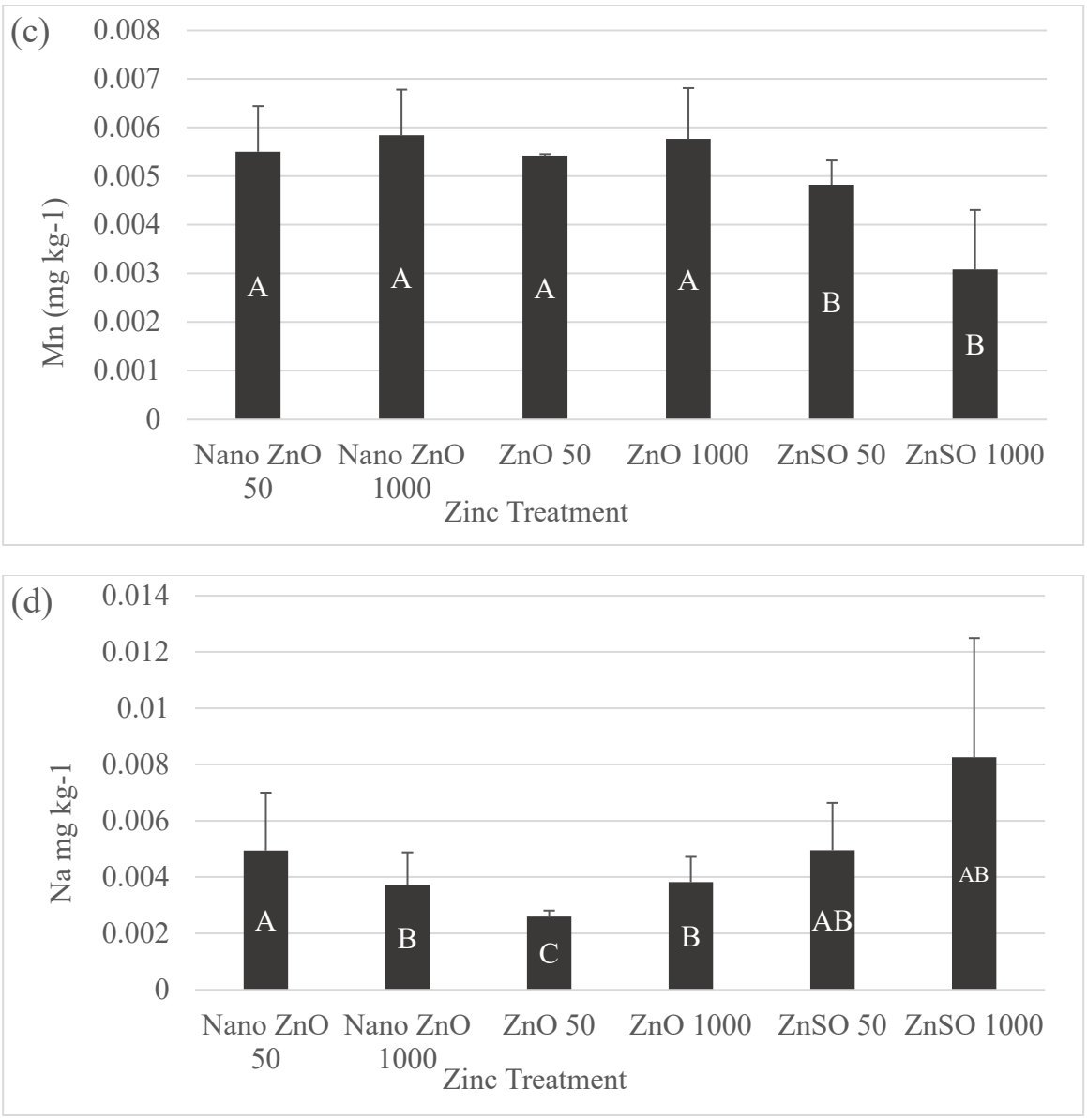

Figure 15. Average concentrations (mg kg^{-1}) of K (a), Mg (b), Mn (c), and Na (d) ± standard deviation in seeds of spring wheat with Nano ZnO 50 mg L^{-1}, Nano ZnO 1000 mg L^{-1}, ZnO 50 mg L^{-1}, ZnO 1000 mg L^{-1}, $ZnSO_4$ 50 mg L^{-1}, and $ZnSO_4$ 1000 mg L^{-1} treatments (continued). Shared letters indicate no significant difference.

Plants exposed to higher concentrations of zinc (in any form) on the leaves had higher concentrations of zinc in seeds ($p = 0.049$) (Figure 16). Though zinc form does not make a difference for the concentrations of zinc in the seeds, zinc oxide NP does affect other elements concentrations in the seeds of spring wheat.

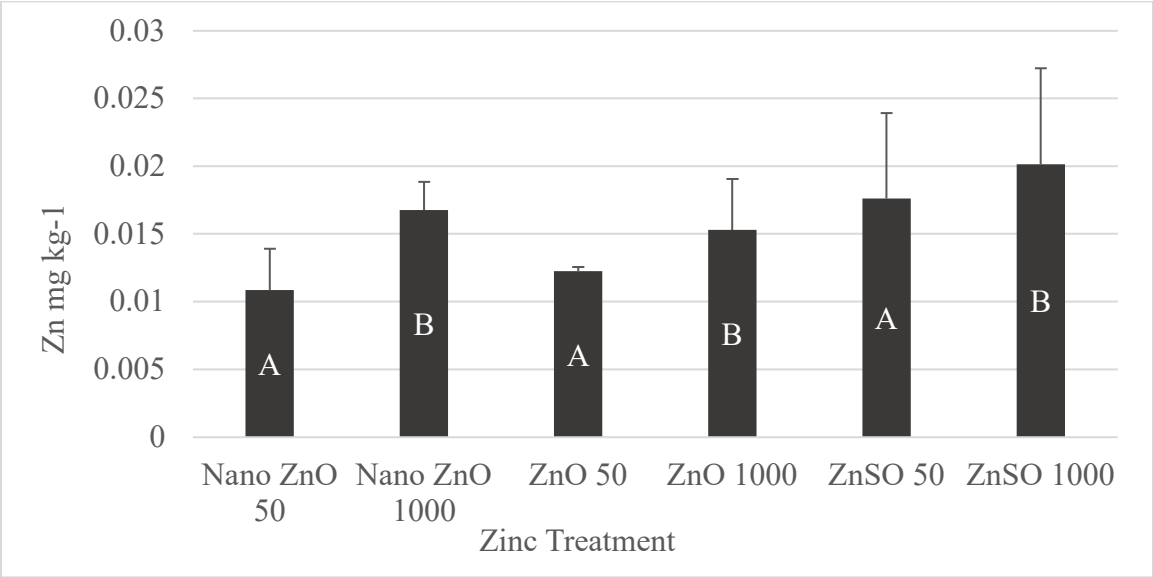

Figure 16. Average concentrations of Zn (mg kg^{-1}) ± standard deviation in seeds of spring wheat with Nano ZnO 50 mg L^{-1}, Nano ZnO 1000 mg L^{-1}, ZnO 50 mg L^{-1}, ZnO 1000 mg L^{-1}, $ZnSO_4$ 50 mg L^{-1}, and $ZnSO_4$ 1000 mg L^{-1} treatments. Shared letters indicate no significant difference.

Table 12. Averages (ave) and standard deviations (sd) for biomass (g) and element concentrations (mg kg^{-1}) in seeds of spring wheat in each treatment. Nano=nanoparticle zinc oxide, ZnO = bulk zinc oxide, ZnSO = zinc sulfate, 50 and 1000 designate concentrations of carbon in nutrient solutions in mg L^{-1}. n=5. The last three columns show the results from a Two-Way ANOVA, P-values, with factors type of zinc, 'Zn-type' (Nano, ZnO, or $ZnSO_4$) concentration, 'Conc' (50 or 1000), and the interaction between the two factors, 'Interaction'. Statistically significant P-values of <0.05 are in bold italics for ease of reading.

Treatment	Nano 50		Nano 1000		ZnO 50		ZnO 1000		ZnSO 50		ZnSO 1000		ANOVA analysis (*p*-values)		
	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	Zn-type	Conc	Interaction
biomass	31.66	18.70	27.70	11.06	23.45	1.20	41.98	10.27	24.18	6.62	15.27	9.49	*0.1240*	*0.7130*	*0.1160*
Al	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.8430*	*0.9850*	*0.5000*
B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.2850*	*0.1430*	*0.4360*
Ca	0.09	0.01	0.10	0.02	0.09	0.03	0.08	0.01	0.10	0.02	0.06	0.02	*0.1810*	*0.1510*	***0.0390***
Fe	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	*0.2700*	*0.0850*	***0.0100***
K	0.95	0.11	0.99	0.23	0.85	0.05	0.94	0.14	1.21	0.14	1.16	0.19	***0.0080***	*0.7310*	*0.7220*
Mg	0.19	0.02	0.20	0.05	0.21	0.04	0.17	0.03	0.22	0.05	0.14	0.03	*0.7690*	***0.0490***	*0.0630*
Mn	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	***0.0060***	*0.4280*	*0.1010*
Mo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.4640*	*0.1110*	*0.4120*
Na	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	***0.0280***	*0.3030*	*0.1790*
P	0.68	0.07	0.73	0.16	0.67	0.06	0.64	0.05	0.78	0.09	0.62	0.07	*0.6670*	*0.0850*	*0.0860*
S	0.25	0.02	0.25	0.04	0.24	0.01	0.25	0.02	0.27	0.01	0.25	0.03	*0.5040*	*0.6840*	*0.6530*
Ti	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.9550*	*0.6930*	*0.5870*
Zn	0.01	0.00	0.02	0.00	0.01	0.00	0.02	0.00	0.02	0.01	0.02	0.01	*0.1030*	***0.0490***	*0.4330*

2.4.2.2. Durum Wheat Seeds

The seeds of durum wheat that were exposed to high concentrations of zinc sulfate solution had lower biomass ($p = 0.036$) (Figure 17, Table 13). Plants exposed to solutions containing zinc sulfate showed the strongest effect of lowered biomass (interaction, $p = 0.024$). Plants exposed to the higher concentrations of zinc sulfate also had significantly higher concentrations of zinc in the seeds ($p = 0.002$). Iron in seeds also varied by the type of zinc treatment with the lowest concentrations in plants exposed to treatments containing zinc sulfate ($p = 0.040$) (Figure 18).

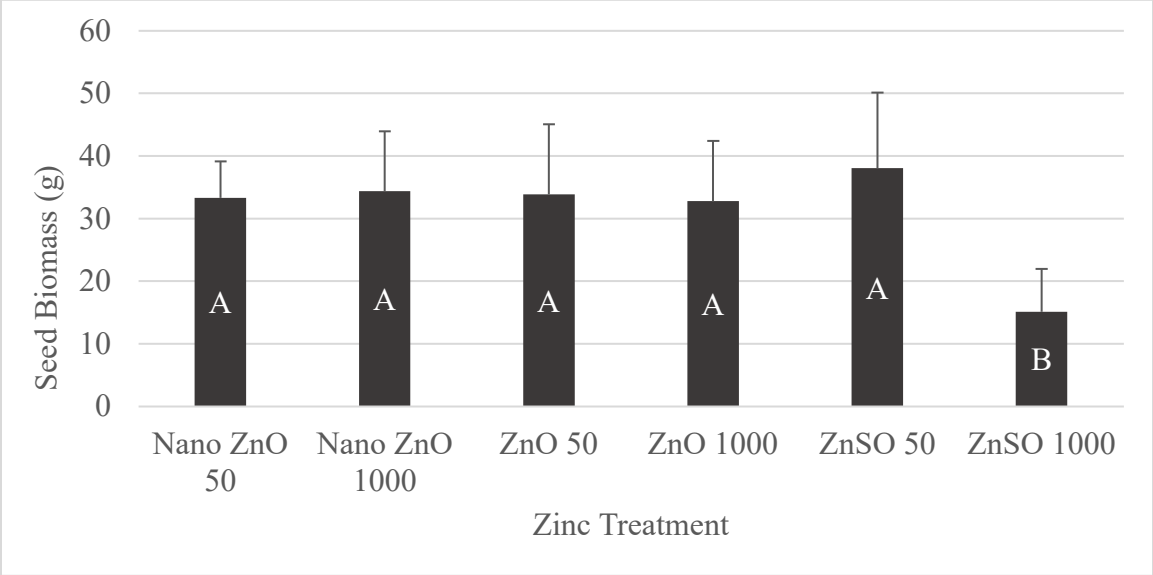

Figure 17**.** Average biomass of seeds (g) ± standard deviation in seeds of durum wheat with Nano ZnO 50 mg L^{-1}, Nano ZnO 1000 mg L^{-1}, ZnO 50 mg L^{-1}, ZnO 1000 mg L^{-1}, $ZnSO_4$ 50 mg L^{-1}, and $ZnSO_4$ 1000 mg L^{-1} treatments . Shared letters indicate no significant difference.

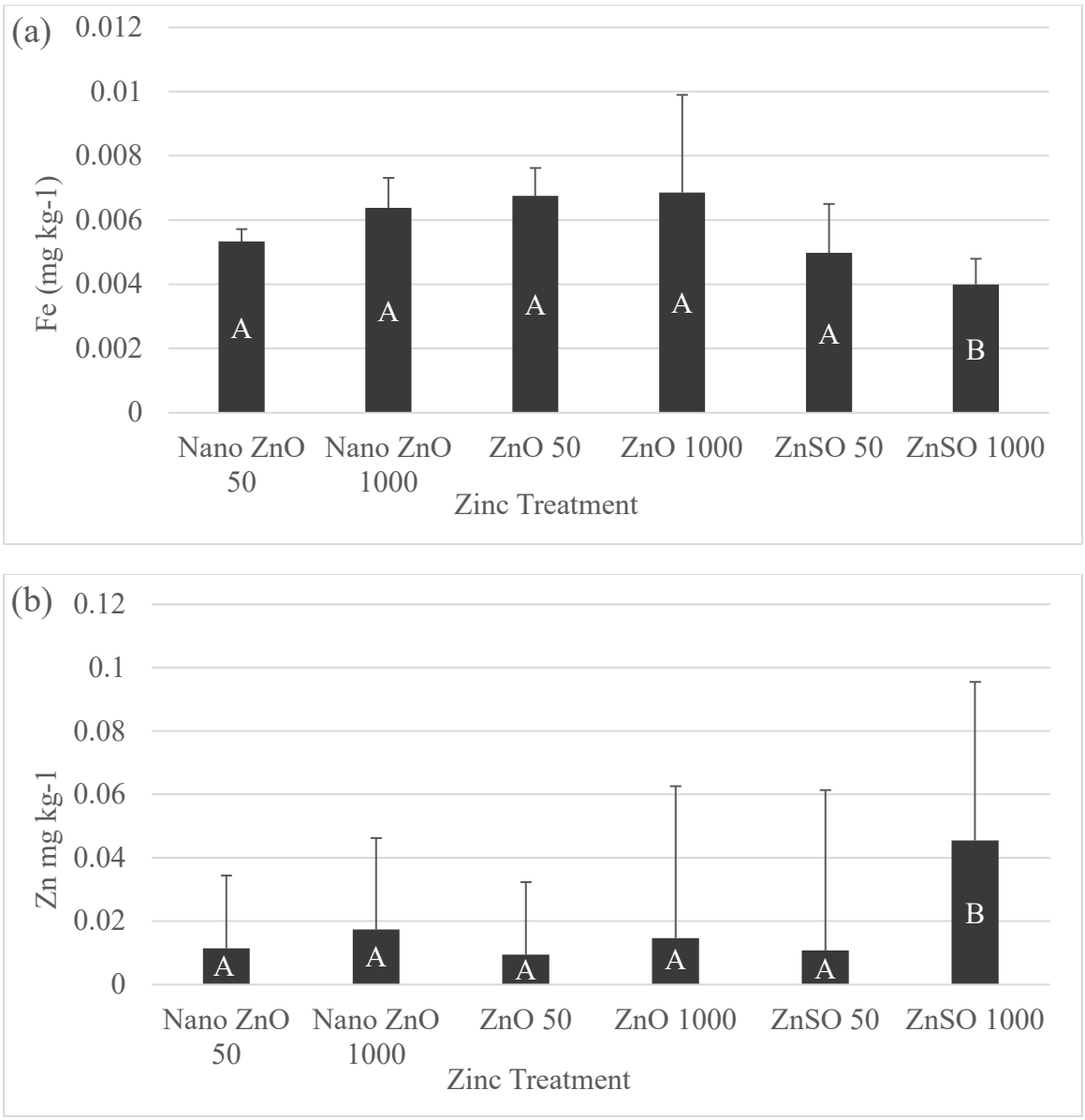

Figure 18. Average concentrations (mg kg^{-1}) of Fe (a), and Zn (b) ± standard deviation in seeds of durum wheat with Nano ZnO 50 mg L^{-1}, Nano ZnO 1000 mg L^{-1}, ZnO 50 mg L^{-1}, ZnO 1000 mg L^{-1}, $ZnSO_4$ 50 mg L^{-1}, and $ZnSO_4$ 1000 mg L^{-1} treatments. Shared letters indicate no significant difference.

Table 13. Averages (ave) and standard deviations (sd) for element concentrations (mg kg^{-1}) in **seeds** of durum wheat in each treatment. Nano=nanoparticle zinc oxide, ZnO = bulk zinc oxide, ZnSO = zinc sulfate, 50 and 1000 designate concentrations of carbon in nutrient solutions in mg L^{-1}. n=5. The last three columns show the results from a Two-Way ANOVA, P-values, with factors type of zinc, 'Zn-type' (Nano, ZnO, or $ZnSO_4$) concentration, 'Conc' (50 or 1000), and the interaction between the two factors, 'Interaction'. Statistically significant P-values of <0.05 are in bold italics for ease of reading.

Treatment	Nano 50		Nano 1000		ZnO 50		ZnO 1000		ZnSO 50		ZnSO 1000		ANOVA analysis (*p*-values)		
	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	Zn-type	Conc	Interaction
Biomass	33.32	6.51	34.40	10.67	33.88	12.94	32.78	11.18	38.08	13.92	15.13	7.95	*0.0870*	***0.0360***	***0.0240***
Al	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.8890*	*0.1340*	*0.4420*
B	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.3110*	*0.4080*	*0.1900*
Ca	0.10	0.02	0.08	0.01	0.09	0.01	0.08	0.02	0.09	0.02	0.12	0.05	*0.3470*	*0.8470*	*0.1900*
Fe	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	***0.0400***	*0.9290*	*0.4600*
K	0.97	0.24	0.87	0.15	1.06	0.14	0.99	0.17	1.01	0.15	1.14	0.49	*0.4220*	*0.9150*	*0.5980*
Mg	0.19	0.03	0.18	0.03	0.21	0.03	0.19	0.06	0.16	0.06	0.18	0.06	*0.5120*	*0.9540*	*0.5410*
Mn	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	*0.0520*	*0.0600*	*0.4910*
Mo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.9210*	*0.0800*	*0.5190*
Na	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.5730*	*0.8540*	*0.6280*
P	0.71	0.11	0.66	0.07	0.70	0.04	0.68	0.08	0.62	0.14	0.70	0.19	*0.8280*	*0.8970*	*0.5070*
S	0.26	0.03	0.25	0.02	0.26	0.02	0.25	0.02	0.24	0.02	0.27	0.11	*0.7250*	*0.9360*	*0.5010*
Ti	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*0.9840*	*0.8520*	*0.7300*
Zn	0.01	0.00	0.02	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.05	0.04	*0.4280*	***0.0020***	*0.7040*

2.5. Discussion

2.5.1. Biomass

Zinc is an essential micronutrient for plant growth and exposure to zinc in varying amounts and forms can significantly influence plant growth and biomass accumulation. In plants exposed to zinc nanoparticles at the roots, the biomass of leaves and seeds from both species of wheat were significantly greater. This increase in biomass was not observed in plants that were exposed to foliar zinc treatments. Sheoran et al. (2021) also observed increased biomass in wheat plants exposed to zinc oxide nanoparticles at the roots. Studies have shown similar results for other plant species including peanut (Prasad et al. 2012), *Arabidopsis* (Wang et al. 2015), carrot (Elizabeth et al. 2017), and rice (Zhang et al. 2019). The larger surface area of zinc oxide nanoparticles increases the ionization rate of zinc oxide nanoparticles which results in more available zinc for uptake by the plant (Mortvedt 1992) and contributes to biomass accumulation (Singh et al. 2018).

2.5.2. Element Uptake/Accumulation

Zinc sulfate is a widely studied zinc compound as it relates to plant uptake and accumulation. Therefore, results for zinc sulfate treatments are somewhat predictable. In the context of these experiments and data analysis, the results for plants treated with zinc sulfate solutions are intended to be used as a general comparison to the zinc oxide treatments, and the focus of the discussion will be on the comparison of the nano and bulk forms of zinc oxide treatments.

2.5.2.1. Root Application – Seeds

Zinc is essential for critical plant functions including metabolic process, enzyme synthesis, protein synthesis, acid synthesis, enzymatic activations, and maintenance of cell

membranes (Marschner 2012). Uptake of bulk zinc ions when exposed at the roots occurs via biotransformation while the uptake of nanoparticles also depends on particle size and surface characteristics (Singh et al. 2018). Zinc oxide nanoparticles have increased surface area and the ionization rate is much higher, which results in more available zinc ions for uptake by the plant. The increased availability of zinc oxide nanoparticles results in increased plant growth. The uptake and accumulation of many other nutrients can also be affected by the increased biomass and also by the altered interactions with nanoparticle zinc oxide.

Magnesium, manganese, and zinc are all considered essential for plant growth (Barker and Pilbeam 2015 and Marschner 2012), and typically exist as divalent cations in the plant substrate environment. Due to their similar chemical compositions, magnesium, manganese, and zinc have all been shown to interact with each other during uptake and accumulation in plants (Barker and Pilbeam 2015).

Magnesium plays a central role in photosynthesis and is also necessary for protein synthesis and enzyme production/activation (Marschner 2012). Demand for magnesium in plants is largely driven by plant growth (Barker and Pilbeam 2015). The results presented here show that with exposure to high concentration zinc treatments, the additional zinc promotes plant growth, and increases the uptake and accumulation of magnesium in the seeds of spring wheat.

Manganese is essential for redox processes, enzyme activations (Marschner 2012), photosynthesis, respiration, protein synthesis (Barker and Pilbeam 2015). Uptake of manganese is dependent on the nutritional status of the plant and the amount of available manganese in the rhizosphere/substrate (Pearson and Renget, 1994). We observed a positive correlation between high concentration zinc treatments and manganese concentration in the seeds of spring wheat. Other studies on the interactions between zinc and manganese show a negative correlation

between zinc and manganese. Authors Singh and Steenberg (1974) showed this antagonistic relationship between zinc and manganese in maize and barley, and it has also been observed in rice (Ishizuka and Ando 1968).

The seeds of durum wheat had higher manganese and potassium concentrations when exposed to low concentration nanoparticles compared to low concentration bulk zinc treatments. This interaction may suggest that nanoparticle zinc enhances the uptake of manganese and potassium when compared with bulk zinc oxide.

2.5.2.2. Root Application - Leaves

Element concentrations in leaves of spring wheat were not significantly affected by exposure to nanoparticle zinc treatments. However, boron, sodium, and phosphorus concentrations were higher in the leaves of durum wheat exposed to nanoparticle zinc treatments. Boron is an essential micronutrient and its uptake in plants is achieved via one of three pathways: passive diffusion, uptake channels, or active carriers. The passive pathway typically occurs under conditions where availability of boron is within the normal range, while the other two pathways typically occur under conditions of boron deficiency or toxicity (Barker and Pilbeam 2015). In maize, boron is taken up and stored in the leaves for later partitioning to the seed (Bender et al. 2013), which could be the same pattern observed here with higher concentrations of boron in the leaves, but not in the seed. Zinc has also been shown to prevent boron toxicity (Hosseini et al. 2007). This may suggest that application of zinc oxide in nanoparticle form may affect the translocation of excess boron to the seed tissue.

Sulphur and zinc have been found to be positively correlated in spring wheat (Cui and Wang 2005) and in mustard (Baudh and Prasad 2012). The uptake of sulphur in plants takes place against a concentration gradient and is energy dependent. Enhanced growth/biomass of

plants that were exposed to the nanoparticle treatments could explain the significantly increased concentrations of sulphur in the seeds.

While sodium is not necessarily an essential plant nutrient, it can affect plant growth and nutrient uptake in various ways. The effects that sodium has on plants can be influenced by many factors including level of exposure, plant species, and substrate composition (Marschner 2012). Like boron, sodium enters plant tissues following concentration gradients (passive transport) and uptake would increase as plants growth increases. The increased leaf biomass also observed with the application of nanoparticle zinc oxide treatments was likely the driver behind increased boron and sodium concentrations in the leaves.

2.5.2.3. Foliar Application - Seeds

Zinc concentrations in seeds of both durum and spring wheat were significantly higher in plants that were treated with high concentration zinc treatments, but there were no significant differences between bulk and nanoparticle treatments. Depkekar et al. (2018) observed increased allocation of zinc in seeds when applied as a foliar spray and that ions from zinc oxide nanoparticles enter the plant via stomatal opening on the surface of the leaf. The results of this study are also consistent with findings reported by Chen (2017) and Cakmak et al (2010). Under the same high concentration zinc treatments, seeds of spring wheat also had higher concentrations of magnesium, while plants exposed to high concentration nanoparticle treatments had higher concentrations of calcium and iron in the seeds. Calcium is an essential macronutrient and is involved in cell signaling in response to environmental stimuli and is essential for maintaining cell walls/lipid membranes. Calcium also can reside in plants in high concentrations without inducing a toxicity effect (Marschner 2012).

Iron can undergo oxidation-reduction reactions which makes it an essential element for plant processes such as metabolic functions and photosynthesis (Barker and Pilbeam, 2015 and Marschner 2012). Zinc/iron and zinc/calcium interactions are well-studied and have been found to have antagonistic interactions. Increasing zinc exposure has been shown to reduce calcium availability (Kalyansundaram and Mehta 1970). Calcium also reduced zinc absorption in rice seedlings (Sadana and Takkar 1985) and barley roots (Kawasaki and Mortisugu 1987), and Davis-Carter et al. (1991) found that fertilizing peanuts with zinc decreased calcium content in the leaves. The uptake of iron is also typically inhibited by zinc (Celik and Katkat 2010, Shahriaripour and Tajabadipour, 2010, and Brar and Sekhon, 1976). Conversely, Morgounov et_al. (2007) found that uptake of zinc and iron were positively correlated in spring and winter wheat when nitrogen supplies were at a high level. The increased concentrations of iron and calcium in the seeds of spring wheat treated with nanoparticle zinc oxide indicates that the nanoparticle form of zinc oxide has a lowered toxicity effect at high concentrations and has altered or reduced interactions with calcium and iron.

2.6. Conclusions

This research aimed to assess the effects of zinc oxide nanoparticles on the element uptake and biomass in wheat plants grown in vermiculite substrate with nutrient solution. Two experiments were conducted to test these hypotheses under varying zinc treatment application methods. For the root exposure experiment - roots of wheat plants were exposed to zinc nanoparticles by applying a zinc nanoparticle solution to the substrate. For the foliar exposure experiment – foliage of wheat plants was exposed to zinc nanoparticles by spraying zinc solutions on the foliage of the plants.

This research aimed to investigate the following hypotheses:

1) Plants exposed to zinc oxide nanoparticles will have increased biomass when compared to plants treated with a bulk form of zinc or no zinc treatment. The results of this experiment showed that biomass of seeds and leaves was significantly greater in plants treated with nano-zinc treatments compared to non-nano zinc treatments when applied to the roots. These results support the hypothesis that plant biomass is significantly greater in plants exposed to zinc oxide nano-particles. Additionally, the nano-zinc treatments had a decreased toxicity effect of high concentration treatments compared to high concentrations of zinc sulfate.

2) Zinc concentrations will be higher in seeds of plants treated with nanoparticle zinc solution. We observed significantly higher concentrations of zinc in plants exposed to higher treatment concentrations, regardless of zinc type. This does not support the hypothesis that plants treated with zinc oxide NP would have increased zinc concentrations in the seeds.

3) Zinc nanoparticle treatments will significantly influence element uptake when compared to bulk zinc treatments. This hypothesis was confirmed by the results of this study. We observed several trends in element uptake/accumulation.

4) that there will be no difference in biomass or element uptake/concentrations between two species of wheat. Most notably, we did not observe increased zinc concentrations in seeds of durum wheat in the root exposure experiment. This may be due to the different growth patterns, where nutrient partitioning/accumulation occurs at different times during the growth trajectory of each species. Otherwise, within each experiment, biomass accumulation and element uptake did not show any notable differences when comparing the two species of wheat.

The results of this study indicate that zinc oxide nanoparticles can affect the biomass and nutrient uptake/accumulation in wheat plants. We observed that root application of zinc oxide

NP can result in increased seed biomass while maintaining proportionate concentrations of zinc in the seeds. This is interesting considering the potential applications of zinc oxide NP as used in fertilizer treatments. Although we observed no significant effects from nano-zinc on plant biomass in the foliar application study, results suggest that various essential elements can be significantly affected by the application of zinc oxide NP, and that the toxicity effects of zinc oxide NP are less than that of zinc sulfate. Further research is needed to understand the interactions more fully between zinc oxide NP and wheat under various growth conditions. At the time of experimental design for this project, studies examining the effects of nanoparticles on plants were mostly limited to short term experiments examining effects on germination or the first stages of plant growth. Therefore, additional studies extending throughout the full plant growth cycle would provide a more complete understanding of the potential effects of zinc oxide NP within the plant system.

3. CARBON NANOTUBES ALTER ELEMENT UPTAKE AND INHIBIT BIOMASS ACCUMULATION IN RICE (*ORYZA SATIVA*)[2]

3.1. Abstract

The continued development of CNTs, especially for use in agriculture, creates opportunities for their release into the environment and biological systems. Because CNTs do not readily biodegrade, they are likely to persist in the environment, especially in soils and aquatic ecosystems. Due to this increasing potential for presence of CNTs within the environment, it is important to gain further understanding as to whether they affect organisms in the environment. The observed effects of CNTs in plant systems have varied and can range from increasing yield/biomass to inducing DNA damage. The present study provides additional information relating to the effects of carbon nanotubes on the growth and nutrient uptake in rice (*Oryza sativa*). Results show that plants treated with CNTs had lower biomass and altered nutrient concentrations in the leaves. These results show that CNTs can affect plant growth/biomass and may also influence element uptake and accumulation in rice.

3.2. Introduction

ENMs possess unique physical, chemical, mechanical, and optical properties. These unique properties are not only driven by the small size of ENMs, but also by the quantum size effect that occurs at the nano-scale (NNI 2019). Changes in properties at the nanoscale vary according to the type of ENMs. For example, the melting point of bulk gold is 1,064 °C but can be as low as 700 °C for gold nanoparticles (Klabunde 2001). Carbon nanotubes (CNT) are highly

[2] This chapter was co-authored by Hannah Passolt, Marinus Otte, Donna Jacob, and Achintya Bezbaruah. Hannah Passolt had primary responsibility for conducting experiments and collecting data, and was the primary developer of the conclusions that are advanced here. Hannah Passolt also drafted and revised all versions of this chapter. Marinus Otte served as proofreader and checked the math in the statistical analysis conducted by Hannah Passolt.

conductive and have a relatively high tensile strength compared with bulk carbon (Dalton et al. 2003). Extensive development of ENMs has led to applications in many industries including computer technology, medical treatments, fertilizers, herbicides, pesticides, food processing/packaging, cosmetics, and industrial coatings (Vithanage et al 2017).

Carbon is a main component of several types of ENMs that are already being used extensively in various industries including computer hardware, sporting goods, medicine, and food processing and packaging. CNTs can be produced using a variety of methods. They consist of a sheet of graphene (one atom in thickness) that has been rolled into a tube (Bethune et al. 1993). The resulting tubes can be of varying diameters, but CNTs are typically one to 100 nanometers in diameter. Single-walled carbon nanotubes (SWCNT) consist of a single sheet of graphene in the shape of a tube with a single wall while multi-walled carbon nanotubes (MWCNT) consist of two or more sheets of graphene.

CNTs are undergoing research and development for use in agriculture and food production systems as components of fertilizers, herbicides, and pesticides (He et al. 2019). These developments can lead to increased efficacy, controlled release, and targeted delivery of fertilizers, herbicides, and pesticides within and near plant systems (Srivastava et al. 2016).

The continued development of CNTs, especially for use in agriculture (Strivastava et al. 2016), creates opportunities for their release into the environment and biological systems. Because CNTs do not readily biodegrade, they are likely to persist in the environment, especially in soils and aquatic ecosystems (Klaine et al. 2008). Due to this increasing potential for presence of CNTs within the environment, it is important to gain further understanding as to whether they affect organisms in the environment. Over time, CNTs may affect plant growth or may be transferred from plants to the food web, and eventually to humans through uptake and bio-

accumulation. Ghosh et al. (2011) found that CNTs can cause damage to DNA in plant and animal cells and Shvedova et al. (2003) observed cell damage and morphological changes in human cells that were exposed to CNTs.

The potential impact of CNTs to plants varies with plant species, nanomaterial type, concentration, and route of exposure. CNTs have been shown to traverse plant cell walls (Yuan et al. 2011), increase water content in germinating seedlings, enhance seed germination (Nair et al. 2012), induce plant cell death (Shen et al. 2010), inhibit root hair elongation (Yan 2013), and alter the conformation of plant DNA (Katti et al. 2015). CNTs may also indirectly impact plant growth by interfering with availability and uptake of organic compounds in the soil (Xia et al. 2010) and Jin et al. (2014) observed altered microbial activity in soils exposed to CNTs.

Because carbon is a natural part of organisms, toxicity of carbon nanoparticles is likely not chemical, but physical, related to the surface properties of nanoparticles. However, scant reports exist on the effect of carbon on uptake and metabolism of nutrients (Nowack and Bucheli 2007, Tiwari et al. 2014, Yang et al. 2013). The main reason for this experiment therefore was to assess the effects of CNTs on element uptake by plants, more specifically rice.

Rice is among the top three crop plants produced worldwide and is considered a staple food source for billions of people around the world. Rice is extensively cultivated and also occurs naturally in some wetland ecosystems. Rice is also being studied for use in remediation of agricultural nutrient runoff (nitrogen and phosphorus) in waters where hyper-eutrophication is an identified issue (Moore et al. 2007, Strivastava et al. 2017).

Previous research into the effects of carbon nanomaterials on rice has shown mixed results. Nair et al. (2012) observed enhanced growth of rice grown in a medium spiked with different types of CNTs at a concentration of 50 µg/mL, while Begum et al. (2014) observed

reduced root and shoot growth in plants exposed to high concentrations of MWCNTs (1000 and 2000 mg/mL hydroponic solutions). Shen et al. (2010) demonstrated that CNTs can induce programmed cell death in rice and the effect was dependent on particle size and dose. Tiwari et al. (2014) investigated the responses of maize (*Zea mays*) seedlings in growth medium with presence of carbon nanotubes found that growth and nutrient uptake were enhanced. However, that study did not include any other forms of carbon addition as controls, and so it could be that the observed effects were not due to carbon in the form of nanotubes, but just an effect of added carbon. In the current study, amorphous activated carbon was chosen as a control to compare with CNTs, in addition to plants being grown in nutrient solution without any added form of carbon in solid form.

As CNTs are released into the environment they can ultimately reside in soils and or water where plants may be exposed to the nanomaterials. Because of the importance of rice as a food crop, its significance in some native plant communities, and the potential for rice to be used for remediation, it is important to understand how exposure to CNTs may interact with and impacted this plant. This research is aimed to investigate the impact of one form of CNT on the growth and nutrient uptake in rice. The hypotheses were as follows: 1) exposure to CNTs will have a negative effect on rice growth (biomass) when compared to plants treated with a bulk form of carbon (activated carbon, AC) or a no-carbon treatment; 2) CNTs interfere with uptake of elements in plant tissue; and 3) there is a stronger effect of CNTs on plant biomass and nutrient uptake with higher concentration carbon treatments.

3.3. Materials and Methods

3.3.1. Experiment Randomization and Setup

The experiment was set up in a greenhouse in October and November, 2015, and used hydroponic solutions for the growth medium. The arrangement of the plants was a randomized block design with six blocks to minimize the effect of slight variations in climatic conditions within the greenhouse. Each block consisted of eight pots with an individual plant, for a total of 48 plants in the experiment. Two of the plants in each block received a no-carbon treatment, consisting of nutrient solution but no added carbon, and the remaining six plants each received one of six carbon treatments as shown in Table 14.

Table 14. Randomized block design of treatments consisting of carbon types (AC=activated carbon, IND CNT = industrial carbon nanotubes, PURE CNT = pure carbon nanotubes), concentrations (0, 50 and 200 mg L^{-1}), and number of replicates.

Concentration	AC	IND CNT	PURE CNT
50 mg L^{-1}	6	6	6
200 mg L^{-1}	6	6	6
0 mg L^{-1} (No Carbon Control)		12	

Activated carbon (AC) was used in this experiment to compare the effects of different carbon types. AC is an amorphous form of carbon that is very porous and has high absorption capabilities (Rajak et al. 2018). It is often used in water purification systems, agricultural applications, and pollutant remediation, which all facilitate the release of AC into the environment. In contrast to AC, carbon in the form of nanotubes has a distinct tubular structure.

This design was chosen for the following reasons:

1. The concentrations for the treatments were based on the ranges of concentrations used in other experiments with nanoparticles and other plant species (0-2000 mg L-1 MWCNTs, Begum and Fugetsu 2012; 20 mg L-1, Yan et al. 2013).

2. The main question of this experiment: “Does exposure to carbon in nanotube form affect uptake of nutrients differently from carbon in an amorphous form?” In other words, if the shape and size of carbon particles mattered in the way carbon affects nutrient uptake by plants. AC treatments therefore were the control treatments relative to the CNT treatments.

3. However, to assess direction of such effects, it was also necessary to have a range of carbon concentrations and including no addition carbon, the 0 mg L-1 treatments. For example, it could be that carbon in any form reduces uptake, but CNTs more than AC. Or maybe AC increases uptake, but CNTs decrease it, and maybe pure CNTs more than industrial CNTs.

4. Additionally, the cost of Pure CNTs is nearly four times higher than IND CNTs. This study will investigate the potential differences or similarities in effects between the two purity levels which is important because IND CNTs are far more accessible for various applications and their release into the environment is more likely with mass production.

5. It was not deemed necessary to have six 0 mg L-1 replicates in each block, which would be more costly and take up more space. Having two in each block provided twelve replicates across the experiment.

3.3.2. Germination and Plant Growth

Experimental units consisted of one plant grown in sand substrate in a mesh bag within a slotted basket suspended in a container made of high-density polyethylene (Figure 19). *Oryza sativa japponica* (rice) seeds were sterilized in a dilute bleach solution (Schwabe et al. 2013) and blotted dry with lint-free paper wipes. Sand, used as a rooting substrate, was passed through a mesh sieve (≥ 0.425 mm) to remove silt and rinsed three times with purified water (RO). For each pot, 300 g of sand were placed in a nylon mesh bag (with pore size smaller than the sand grain size) that was supported in a plastic slotted basket and then placed into a solid black 0.95-

liter HDPE thermoplastic container. Three seeds were planted simultaneously in each pot assembly to ensure the successful germination of at least one seed per pot. The seeds were planted at a depth of about 1.2 cm, with approximately 2.5 cm between each seed. After 14 days of growth, seedlings were thinned to one plant per pot, based on uniformity of size (two leaves emerged and approximately 2.5 cm tall), and the water was replaced with nutrient solution (Yoshida 1976), see Table 15. The Yoshida nutrient solution was selected because it was designed specifically for hydroponic experiments with rice. The container was filled with 800 mL of nutrient solution in order to saturate the sand substrate but not submerge the plants. Nutrient solution was changed twice a week.

Table 15. Composition of Yoshida nutrient solution (1976).

Stock Solution	**Element**	**Reagent**	**Preparation (g per 10 L RO water)**		**Final Concentration (mg L^{-1})**
1	N	NH_4HNO_3	914		40
2	P	$NaH_2PO_4 \cdot 2H_2O$	403		10
3	K	K_2SO_4	714		40
4	Ca	$CaCl_2$	886		40
5	Mg	$MgSO_4 \cdot 7H_2O$	324		40
6	Mn	$MnCl_2 \cdot 4H_2O$	15.0	Dissolved separately, then combine with 500 ml of concentrated H_2SO_4. Make up to 10 L volume with RO water	0.5
	Mo	$(NH_4)_6 \cdot MO_7O_{24} \cdot 4H_2O$	0.74		0.05
	B	H_3BO_3	9.34		0.20
	Zn	$ZnSO_4 \cdot 7H_2O$	0.35		0.01
	Cu	$CuSO_4 \cdot 5H_2O$	0.31		0.01
	Fe	$FeCl_3 \cdot 6H_2O$	77.0		2.00
		Citric acid (monohydrate)	119		NA

To make 4 liters of nutrient solution:
Add 5 milliliters of each stock solution to 1 liter of DI water. Stir after each addition.
Adjust the pH of the solution to 5.0 by slowly adding 1 N NaOH, stirring continuously.
Add 1 liter of this solution to 3 liters of RO water, or 200 mL concentrated nutrient solution to 600 mL of RO water for each 800 mL per container.

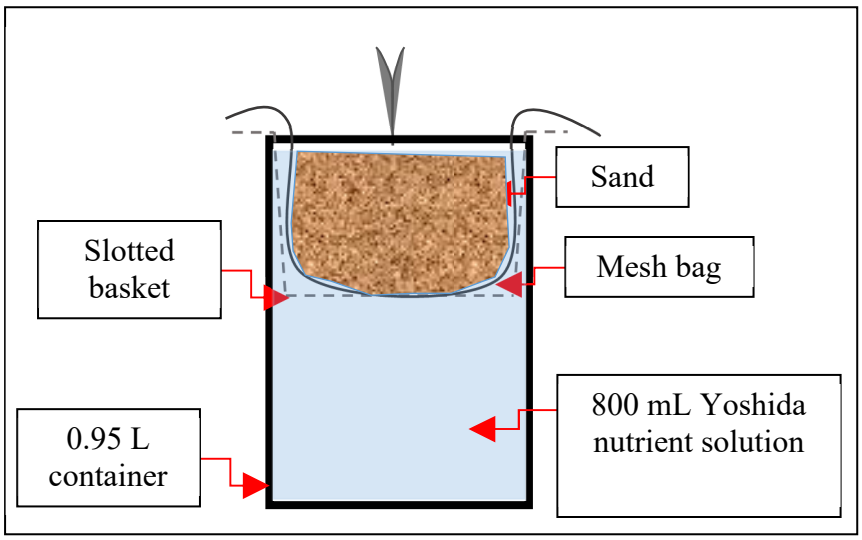

Figure 19**.** Diagram of an experimental unit.

3.3.3. Carbon Treatment Method

After 21 days of growth, a single application of concentrated carbon treatments was added to the surface of the sand during the process of changing the nutrient solution. Carbon nanotubes were obtained from Nanostructures and Amorphous Materials, Inc. (www.nanoamor.com). The CNTs utilized in this project had an outside diameter of less than two nanometers and length of 5-30 nanometers. The Industrial CNTs (IND CNT) have a purity rating of 60 %, indicating they contain higher amounts of residual catalyst, ash, or carbon from the production process. The Pure CNTs have a purity rating of 95 %, and thus, a much lower amount of the additional substances that are present in IND CNTs.

Each container held 800 mL of nutrient solution. The nutrient solution was made by concentrated stock solution (200 mL concentrate added to 600 mL RO water). When the carbon treatments were added, 100 mL of treatment solution (carbon and RO water) were incorporated with the 600 mL RO water during nutrient solution preparation to achieve the same concentrations of nutrients. The proportions of stock solution to RO water remained the same,

but with the addition of either 0, 50 or 200 mg C L^{-1} as industrial CNT, pure CNT, or AC. The homogenous solution was applied evenly to the surface of the sand in each container.

The plants grew from October 7 to November 25th, 2015 in the sand and nutrient solution with carbon treatments under 16 h photoperiod and temperatures ranging between 20 and 30°C. Nutrient solution was changed once a week until the third leaf emerged on the 80 % of seedlings. After this point, nutrient solution was changed two times per week. Because algae began growing on the pot assembly, steps were taken to mitigate algae growth. The plant containers (black plastic pots) were replaced with clean containers, after which a 1:1 hydrogen peroxide and RO water solution was sprayed onto any surfaces with algae to eliminate algal growth on each plant (Barrington and Ghadouani 2011). During this application plants were shielded using a sheet of plastic to avoid tissue damage by direct contact with the hydrogen peroxide solution.

3.3.4. Plant Harvest

Plants were harvested before flowering, ten weeks after planting (September 16th, 2015 to November 25th, 2015). Each plant was removed carefully from the sand substrate and the mesh bag and rinsed with water to remove sand, then blotted dry with clean paper towels. If roots were entangled in the mesh bag and would not loosen easily, they were cut from the outside of the bag to release the rest of the root-mass from inside the mesh bag. The separated roots were kept with their respective plants for further processing. Above-ground biomass (stems and leaves combined) was separated from the below-ground biomass (roots) and weighed.

3.3.5. Sample Preparation and ICP Analysis

For element analysis, the same method was used as for Kissoon et al. (2011), except that nitric acid was used for digestion of plant materials instead of sulfuric acid, as follows. The

difference was that sulfuric acid dissolves titanium which was the element of focus in that study. However, sulfuric acid forms insoluble precipitates with elements like calcium and barium, which were of interest in this study. That problem is avoided with nitric acid, but it will not dissolve titanium very well. Titanium was therefore not included in the statistical analysis for this study. The separated above-ground and below-ground portions were placed in paper bags and dried at 60 °C for seven days until no further weight change was observed. After drying, the leaves and roots of each plant were separated and weighed, then ground to a fine powder using liquid nitrogen in a mortar and pestle. The samples, as well as triplicates of a certified reference plant material (CRM, NCS DC 73350 poplar leaves, China National Analysis Center of Iron and Steel), were predigested in 5 ml concentrated HNO_3 (samples <250 mg were predigested in 3 ml HNO_3) for at least 16 hours in Xpress 55 ml PFA venting vessels, after which 5 ml of ultrapure DI water was added. The samples were then digested using a CEM Mars Xpress microwave digester at 200 °C for 25 minutes with a 25-minute ramp to heat program.

Digested samples were analyzed via ICP-OES (Thermo iCAP 6000) at The Connecticut Agricultural Experiment Station in New Haven, CT. The following 22 elements were analyzed: Al, B, Be, Ca, Cd, Ce, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Ti, and Zn. Continuing calibration verification (CCV) checks containing 22 elements were performed after every twelve samples. The detection limits for each element are shown in Table 16.

Table 16**.** Elements analyzed via ICP and detection limit (DL) in mg L^{-1} of liquid digest.

Element	**DL (mg L^{-1})**	**Element**	**DL (mg L^{-1})**
Al	0.006	Mg	0.002
B	0.002	Mn	0.004
Be	0.002	Mo	0.003
Ca	0.005	Na	0.005
Cd	0.002	Ni	0.002
Ce	0.004	P	0.005
Co	0.003	Pb	0.007
Cr	0.001	S	0.005
Cu	0.279	Se	0.010
Fe	0.004	Ti	0.001
K	0.049	Zn	0.197

3.3.6. Data Analysis

Statistical analysis of the data followed procedures recommended by Reimann et al. (2008). Elements for which greater than fifty percent of the sample concentrations were below the detection limit (censored values) were excluded from further analysis. Of the remaining elements, values that were below the specified detection limit were given a value of half of their detection limit for the purpose of data analysis. Blanks were averaged and the concentration subtracted from each sample concentration. Statistical analysis was performed with Minitab 18. Distributions for the data for each element were checked for normality and if needed were transformed using a Box-Cox or Johnson transformation, as determined by the Individual Distribution Identification module in Minitab 18. Data were tested by Two-Way ANOVA, in which two of each of the No-carbon treatments were randomly assigned to the three types of carbon treatments (AC, IND CNT and PURE CNT), and three concentrations (0, 50 and 200 mg L^{-1}).

3.4. Results

3.4.1. Biomass

The average values of biomass and element concentrations for roots and shoots for each treatment are summarized in Tables 17 and 18. Biomass was highly variable, as is indicated by the large standard deviations relative to the averages. Biomass of both roots and shoots were affected by the type of carbon (Figure 20), but not by their concentrations, nor were there significant interactions. Biomass of the roots (Table 17) in the No-carbon and the AC treatments were similar with dry weights around 0.4 g. The dry weights of the two CNT treatments were similar, but an order of magnitude lower than the other treatments with averages ranging from 15.0-41.0 mg. Biomass of the shoots showed similar patterns, was generally higher than the roots, with average values ranging from 0.1 to 1.3 g in No-carbon and AC treatments, but in the CNT treatments ranging from 53.0 to 99.0 mg, see Figure 20 and Table 18.

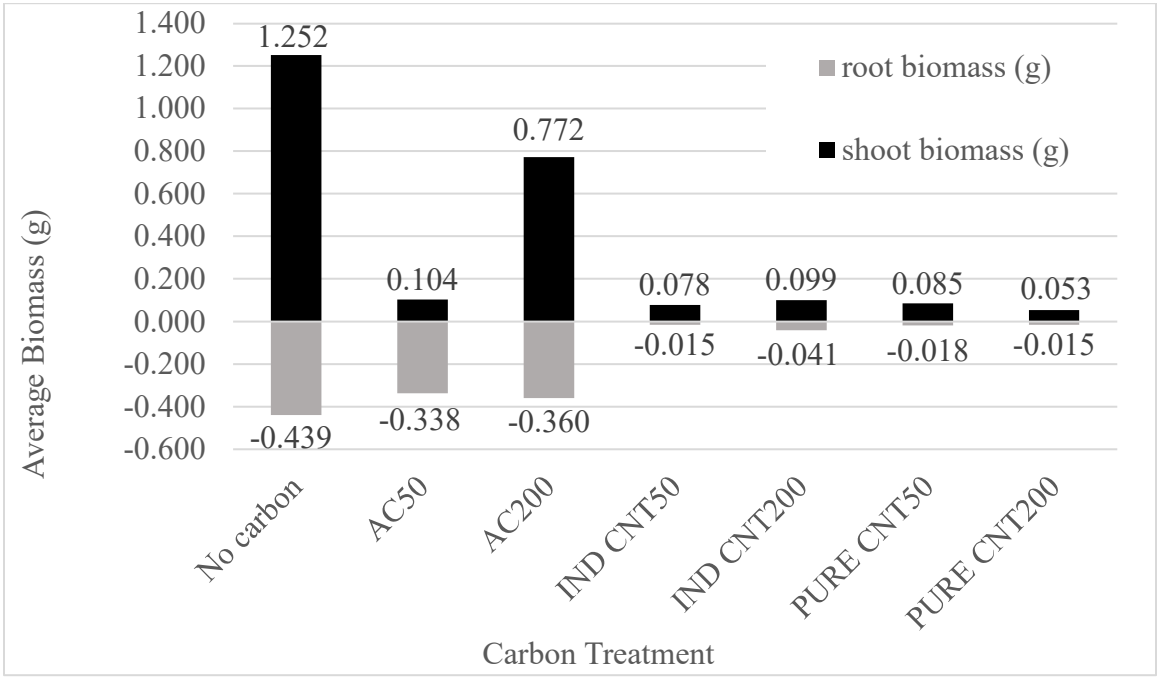

Figure 20**.** Average biomass of shoots and roots (as negative values) of rice plants treated with just nutrient solution (No-carbon), activated carbon (AC), industrial carbon nanotubes (IND CNT) or pure carbon nanotubes (PURE CNT) at either 50 or 200 mg L^{-1}.

Table 17. Averages (ave) and standard deviations (sd) of biomass and element concentrations (mg kg^{-1}) in **roots** of rice in each treatment. AC=activated carbon, IND CNT = industrial carbon nanotubes, PURE CNT = pure carbon nanotubes, 50 and 200 designate concentrations of carbon in nutrient solutions in mg L^{-1}. The No-carbon treatment contained just nutrient solution. *n*=6, except for No-carbon, which was *n*=12. The last three columns show the results from a Two-Way ANOVA after appropriate transformation, either log or Box-Cox, two of the twelve No-carbon treatments randomly assigned to each C-type *p*-values, with factors type of carbon, 'C-type' (AC, IND CNT or PURE CNT), concentration, 'Conc' (0, 50, or 200), and the interaction between the two factors, 'Interaction'. Statistically significant *P*-values of <0.05 are in ***bold italics*** for ease of reading.

Treatment	No carbon		AC50		AC200		IND CNT50		IND CNT200		PURE CNT50		PURE CNT200		(*P*-values)		
	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	C-type	Conc	Interaction
Biomass	0.440	1.290	0.340	0.720	0.360	0.590	0.015	0.005	0.041	0.054	0.018	0.010	0.015	0.006	***0.000***	*0.953*	*0.538*
Al	3.400	3.600	4.700	3.200	8.000	9.300	1.600	0.700	3.400	5.200	1.700	1.900	1.700	1.300	*0.866*	*0.347*	*0.302*
B	0.024	0.022	0.031	0.023	0.041	0.037	0.010	0.004	0.016	0.011	0.011	0.005	0.011	0.006	*0.108*	*0.285*	*0.213*
Ca	18.200	13.900	22.100	17.600	40.700	42.000	9.500	7.200	15.900	22.100	12.300	12.200	6.900	4.100	*0.301*	*0.175*	*0.389*
Co	0.013	0.013	0.013	0.007	0.035	0.038	0.008	0.004	0.010	0.010	0.007	0.003	0.006	0.003	*0.800*	*0.900*	*0.242*
Cr	0.019	0.014	0.027	0.016	0.033	0.036	0.009	0.004	0.015	0.016	0.009	0.008	0.010	0.005	*0.087*	*0.106*	***0.023***
Fe	12.900	10.100	14.600	7.200	19.800	20.700	6.200	2.100	9.400	8.200	6.500	6.700	5.800	3.100	*0.226*	*0.120*	*0.110*
K	50.500	99.500	74.400	66.600	96.000	107.10	12.200	12.600	33.900	53.100	9.000	3.700	6.600	3.000	***0.016***	*0.496*	*0.987*
Mg	11.200	13.500	18.700	16.900	28.000	28.400	4.700	3.400	7.800	10.700	3.500	2.900	2.500	1.400	*0.516*	*0.414*	*0.543*
Mn	1.180	1.420	1.280	0.650	3.370	3.030	0.780	0.490	0.810	0.610	0.530	0.160	0.580	0.140	*0.992*	*0.379*	*0.170*
Na	20.000	39.700	32.800	59.300	31.900	42.300	4.200	1.900	5.200	3.800	3.900	1.500	3.700	0.900	*0.279*	*0.974*	*0.638*
P	17.200	24.300	19.000	17.500	32.600	31.800	6.000	3.400	9.700	9.800	5.500	1.500	4.500	1.700	*0.313*	*0.787*	*0.111*
S	17.600	30.500	24.900	36.700	38.700	46.600	3.300	2.500	7.200	8.900	2.700	0.500	2.400	0.800	***0.015***	*0.620*	*0.821*

3.4.2. Element Uptake/Accumulation in Roots

Concentrations of Be, Cd, Ce, Cu, Mo, Ni, Pb, Se, Ti, and Zn were below the detection limit for greater than 50% of the observations and were therefore not included in data analysis and will not be further discussed.

In the roots (Table 17), concentrations of Ca, Fe, K, Mg, Na, P, and S were present in the same order of magnitude, generally between 1 and 100 mg kg^{-1}. Concentrations of Al and Mn were an order of magnitude lower, generally between 0.1 and 8 mg kg^{-1}. The concentrations of B, Co, and Cr were much lower again, with concentrations between 0.009 and 0.41 mg kg^{-1}. The concentrations of K and S were affected significantly by carbon type, with values in both CNT treatments much lower than in the No-carbon or AC treatments (Figure 21). Neither carbon type nor the interactions between the two factors significantly affected any of the element concentrations, except for Cr (Figure22). For that element, the main factors did not significantly affect concentrations, but the interaction was significant. This is clear from Figure 22, which shows that concentrations in AC treatments were higher than in the No-carbon treatments, but lower in both CNT treatments. In addition, concentrations of Cr were higher in the 200 mg L^{-1} treatments than in the 50 mg L^{-1} treatments for all types of carbon.

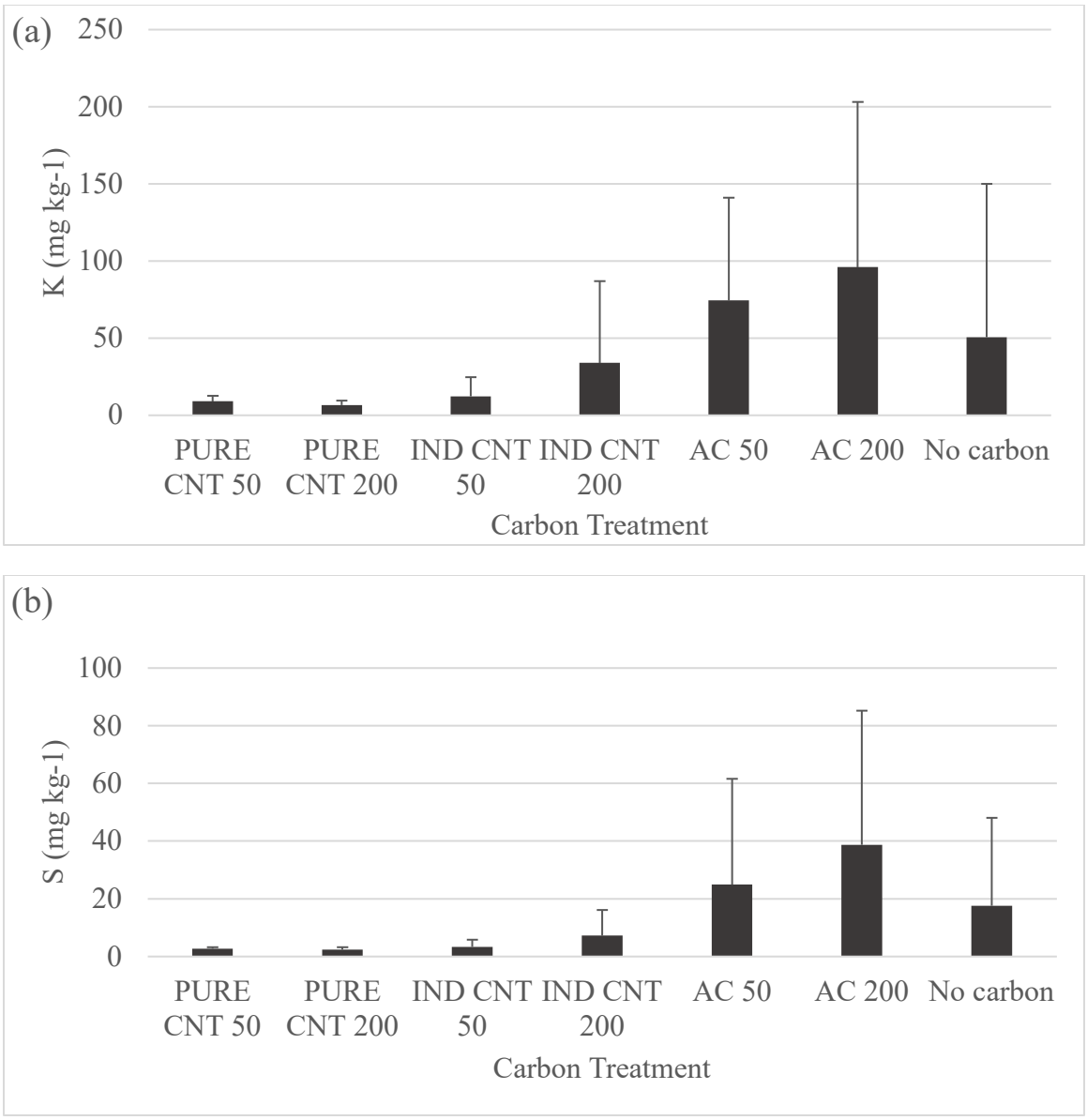

Figure 21. Average concentrations (mg kg^{-1}) of K (a) and S (b) in the roots of rice plants treated with just nutrient solution (No carbon), activated carbon (AC), industrial carbon nanotubes (IND CNT) or pure carbon nanotubes (PURE CNT) at either 50 or 200 mg L^{-1}.

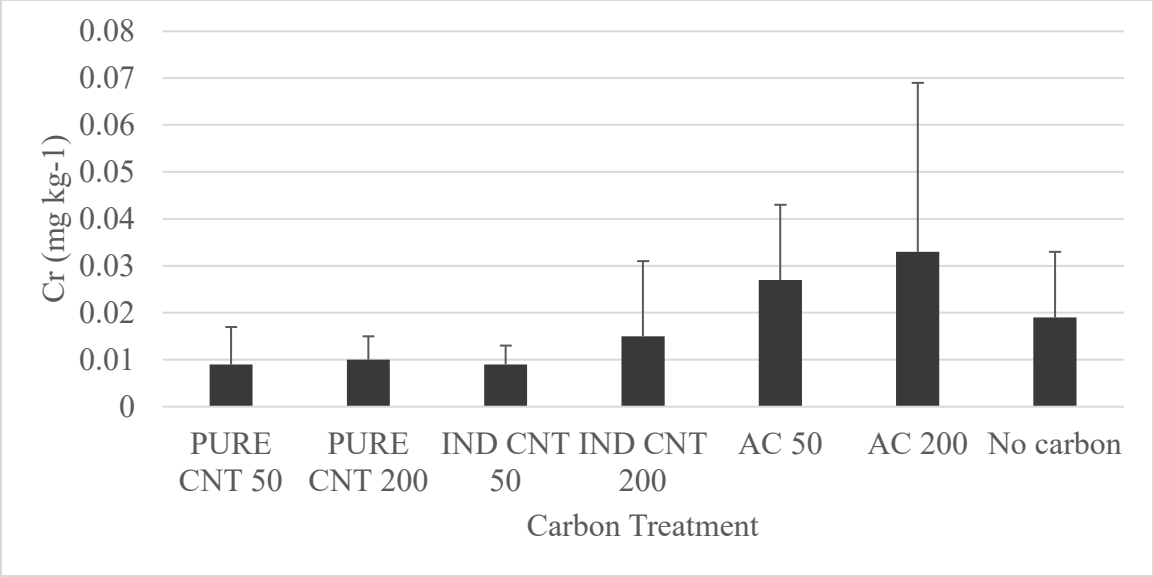

Figure 22. Average concentrations (mg kg^{-1}) of Cr in the roots of rice plants treated with just nutrient solution (No carbon), activated carbon (AC), industrial carbon nanotubes (IND CNT) or pure carbon nanotubes (PURE CNT) at either 50 or 200 mg L^{-1}.

3.4.3. Element Uptake/Accumulation in Shoots

Concentrations of Be, Cd, Ce, Cu, Mo, Ni, Pb, Se, Ti, and Zn were below the detection limit for greater than 50% of the observations and were therefore not included in data analysis and will not be further discussed.

In the shoots, concentrations of K were particularly high compared to other elements, ranging from about 100 to 300 mg kg^{-1}, and on average about eight times higher than in the roots. Concentrations in the shoots of Ca, Mg, Na, P and S were about ten times lower than those of K, at about 20 to 75 mg kg^{-1}, and about two to eight times higher than in the roots. Concentrations of Fe and Mn were another order of magnitude lower at about 0.8 to 4.6 mg kg^{-1}. But Fe concentrations in the shoots were much lower than in the roots, with an average shoot/root ratio of about 0.15, while those of Mn were higher at a shoot/root ratio of about 4.3. Al and B concentrations were another order of magnitude lower at about 0.17 to 0.68, and Co and Cr yet another magnitude lower at 0.002-0.003 mg kg^{-1}. However, the concentrations of B in

the shoots were much higher than in the roots, about 21 times, while the other concentrations were lower with a shoot/root ratio of about 0.15 to 0.27.

Concentrations of B, Ca, and Na were further significantly affected by the type of carbon (Table 18), but not by differences in concentrations, nor were any of the interactions significant. Concentrations of B were on average higher in the AC treatments compared to both CNT treatments (Figure 23), which was a similar pattern as observed for Ca (Figure 24). However, concentrations of Na (Figure 25) on average were lower in the IND CNT treatments compared to either the AC or PURE CNT treatments.

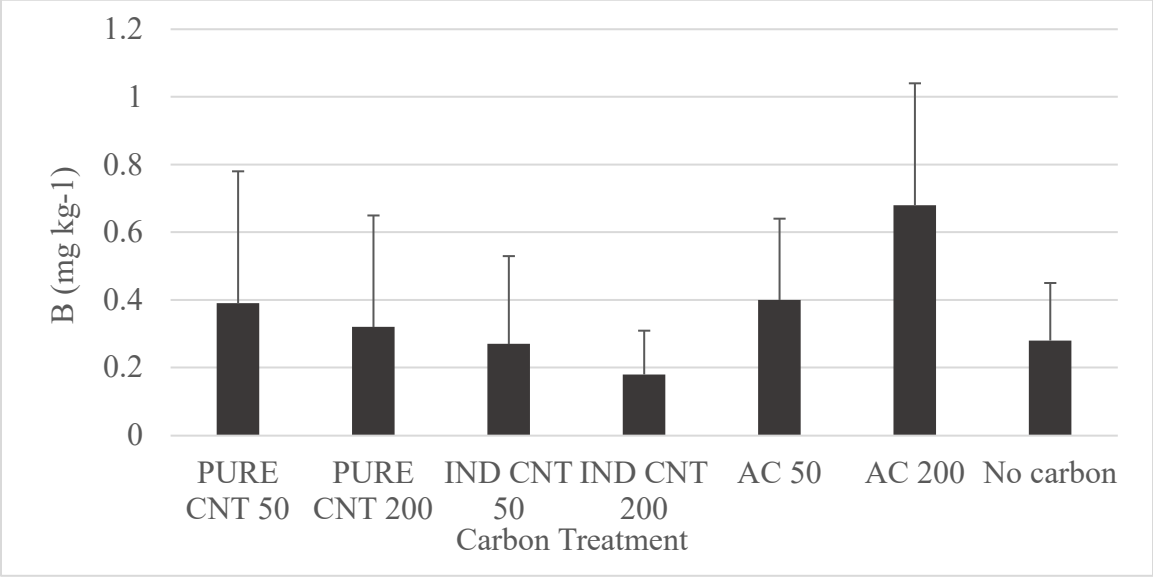

Figure 23. Average concentrations (mg kg^{-1}) of B in the shoots of rice plants treated with just nutrient solution (No carbon), activated carbon (AC), industrial carbon nanotubes (IND CNT) or pure carbon nanotubes (PURE CNT) at either 50 or 200 mg L^{-1}.

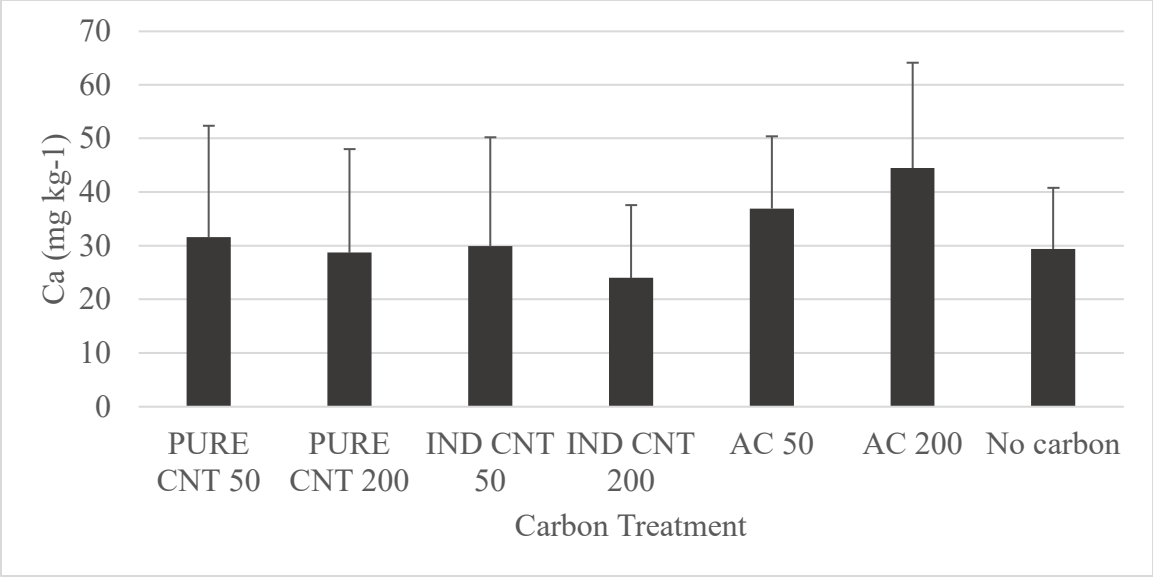

Figure 24. Average concentrations (mg kg^{-1}) of Ca in the shoots of rice plants treated with just nutrient solution (No carbon), activated carbon (AC), industrial carbon nanotubes (IND CNT) or pure carbon nanotubes (PURE CNT) at either 50 or 200 mg L^{-1}.

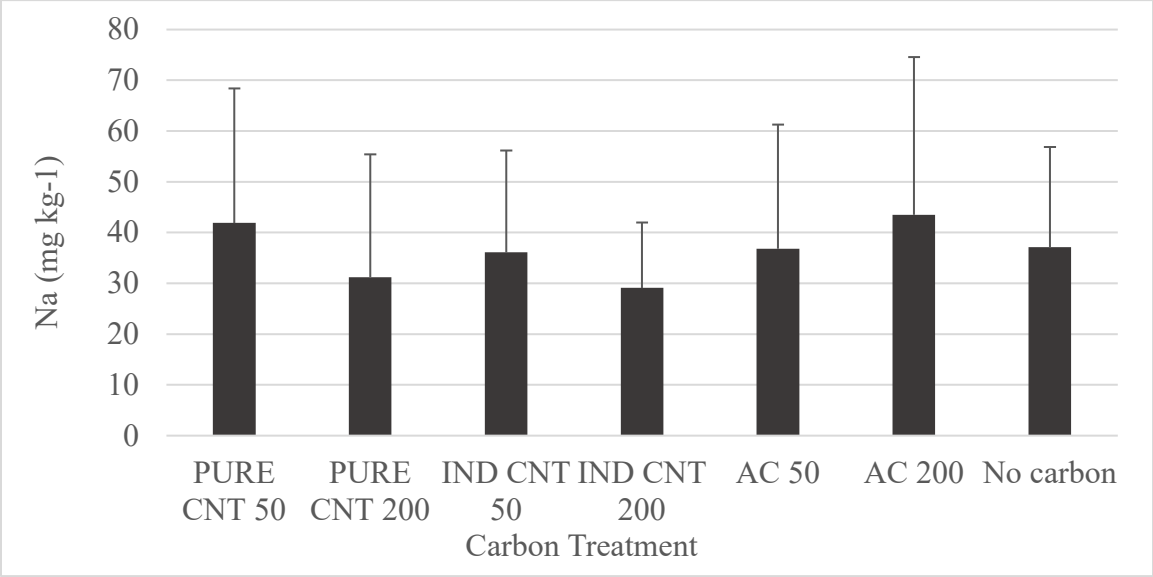

Figure 25. Average concentrations (mg kg^{-1}) of Na in the shoots of rice plants treated with just nutrient solution (No carbon), activated carbon (AC), industrial carbon nanotubes (IND CNT) or pure carbon nanotubes (PURE CNT) at either 50 or 200 mg L^{-1}.

Table 18. Averages (ave) and standard deviations (sd) for element concentrations (mg kg^{-1}) in **shoots** of rice in each treatment. AC=activated carbon, IND CNT = industrial carbon nanotubes, PURE CNT = pure carbon nanotubes, 50 and 200 designate concentrations of carbon in nutrient solutions in mg L^{-1}. The No-carbon treatment contained just nutrient solution. n=6, except for No-carbon, which was n=12. The last three columns show the results from a Two-Way ANOVA, p-values, with factors type of carbon, 'C-type' (AC, IND CNT or PURE CNT) concentration, 'Conc' (0, 50, or 200), and the interaction between the two factors, 'Interaction'. Statistically significant P-values of <0.05 are in ***bold italics*** for ease of reading.

Treatment	No carbon		AC50		AC200		IND CNT50		IND CNT200		PURE CNT50		PURE CNT200		(*P*-values)		
	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	ave	sd	C-type	Conc	Interaction
Biomass	1.300	3.000	0.100	0.050	0.770	0.300	0.078	0.070	0.099	0.133	0.085	0.078	0.053	0.043	***0.000***	*0.836*	*0.609*
Al	0.420	0.330	0.570	0.440	0.470	0.290	0.300	0.250	0.460	0.600	0.230	0.230	0.450	0.240	*0.273*	*0.416*	*0.165*
B	0.280	0.170	0.400	0.240	0.680	0.360	0.270	0.260	0.180	0.130	0.390	0.390	0.320	0.330	***0.027***	*0.459*	*0.662*
Ca	29.400	11.40	36.90	13.50	44.50	19.60	29.90	20.30	24.000	13.600	31.600	20.800	28.700	19.300	***0.025***	*0.592*	*0.926*
Co	0.003	0.003	0.003	0.002	0.003	0.003	0.003	0.003	0.003	0.005	0.002	0.002	0.002	0.002	*0.817*	*0.917*	*0.441*
Cr	0.003	0.003	0.003	0.002	0.002	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.005	0.004	*0.165*	*0.465*	*0.180*
Fe	1.300	0.800	2.100	1.800	1.200	0.400	0.900	0.500	0.800	0.700	1.400	2.200	2.000	1.600	*0.071*	*0.591*	*0.247*
K	143.50	106.50	184.30	87.00	289.70	144.20	134.80	138.80	117.10	142.20	151.50	121.40	104.50	118.50	*0.187*	*0.794*	*0.219*
Mg	24.300	14.300	28.500	15.300	44.400	21.600	19.200	16.400	18.000	12.400	26.200	23.700	21.800	18.800	*0.060*	*0.233*	*0.873*
Mn	3.740	2.960	4.570	1.550	8.300	3.980	3.590	2.450	2.490	2.190	3.980	3.910	3.450	3.650	*0.496*	*0.289*	*0.319*
Na	37.100	19.800	36.800	24.500	43.500	31.100	31.200	24.200	29.100	12.900	41.900	26.500	36.100	20.100	***0.021***	*0.310*	*0.583*
P	34.500	24.400	46.700	21.100	75.000	37.900	32.000	28.800	31.000	33.700	40.600	37.400	22.000	17.600	*0.341*	*0.318*	*0.107*
S	35.100	18.400	46.000	23.700	71.200	37.800	29.500	24.200	33.400	26.300	40.700	37.500	24.300	13.100	*0.337*	*0.192*	*0.654*

3.5. Discussion

3.5.1. Biomass

Root and shoot biomass in both the CNT treatments were significantly lower compared to the No carbon or AC treatments. Other studies have also found negative effects of CNTs on plant growth. Begum et al. (2012) observed that red spinach (*Amaranthus dubius*) exposed to CNTs had decreased biomass of the roots and shoots compared with plants receiving no nano carbon, though the effects described by Begum et al. (2012) were particularly evident at concentrations higher than used in this study, up to 1000 mg L^{-1}. In contrast, Lin et al. (2007), testing several plant species including the grasses maize (*Zea mays*) and ryegrass (*Lolium perenne*), found no effects on growth of plant roots at concentrations of 2000 mg L^{-1}. In turn, Liu et al. (2009) have shown that CNTs can penetrate plant cell walls and translocate to other plant parts. Yuan et al. (2011) showed that CNTs can pass mesophyll cell walls and membranes of *Arabidopsis* protoplasts and negatively affect plant physiology. It appears therefore that carbon nanotubes can be taken up by plants, translocated into the shoots, and cause damage at high exposures. This likely explains the lower biomass of the rice plants in this study grown on CNTs compared to those grown on AC or on just nutrient solution.

3.5.2. Element Uptake

The rice plants showed changes in uptake of some elements in response to differences in amounts and types of carbon in the growth medium. The roots accumulated higher concentrations of K and S in the AC treatments, but lower concentrations in the CNT treatments, compared to the No carbon treatment. This could not be explained by dilution/concentration effects, because the root biomass showed similar patterns. This then suggests that the carbon in

the CNTs affected the roots differently than AC, inhibiting growth and reducing accumulation of K and S in the roots.

The pattern for Cr in roots was similar to that of K and S, but here the interaction between type of carbon and concentration in the growth medium was significant, suggesting that the magnitude of the effect of concentration depended on the type of carbon.

In the shoots, AC increased concentrations of B, Ca, and Na compared to the No carbon treatment, but IND CNT particularly led to lower concentrations. The overall conclusion then is that C in the form CNTs showed some level of toxicity by reducing rice plant growth and changing uptake or translocation of some nutrients.

Boron is necessary for several plant functions, and the uptake of boron can be dependent on the presence of other nutrients – especially calcium, which is also essential for plant growth (Siddiqui et al. 2013). This may explain the similar patterns in concentrations of B and Ca in response to the treatments.

Pandey et al. (2018) observed that CNTs absorbed sodium ions by ionic bonds from a NaCl solution thereby reducing the activity of Na in solution, which may have explained observed responses to salt stress in soils, thus improving growth of sorghum (*Sorghum spp.*) and switchgrass (*Panicum virgatum*). Reduced activity of Na in the nutrient solution due to CNTs in this experiment may therefore also explain the lower concentrations in the shoots.

Very few reports exist about the effects of carbon, amorphous as activated carbon or in the form of nanotubes, on uptake of elements in plants. Yu et al. (1993) grew tomatoes in nutrient solution and concluded that their weight and fruit yield increased due to addition of activated carbon, but that "No appreciable changes were brought about in the concentrations of major and trace elements both in the solution and in the plant by the addition of activated

charcoal". Studying effects of activated carbon (AC) on cucumbers, Hilber et al 2009 concluded that "AC soil treatments did neither affect the availability of nutrients to the cucumber plants nor their yield". So, whether in nutrient solution, as in this study, or in soil, activated carbon addition was not reported to affect plants, but in this study it did. Of course, because pure carbon added to nutrient solution in any form does not dissolve, interactions must be mostly physical, rather than chemical. The effects of carbon nanotubes on plants were reviewed by Vithanage et al. (2017) showing that research is expanding, but that information is scattered and often contradictory. The results from this study, however, do suggest that it matters in what form carbon is added, even, as in the case of B, if nanoparticles are 'pure' or not. The effects of CNTs on nutrient uptake in plants need to be further investigated, as the few reports that exist on effects of nanoparticles on element uptake by plants are unclear on this issue. Tiwari et al. (2014) investigated the effects of addition of carbon nanotubes to growth medium in the form of agar on maize (*Zea mays*) seedlings, and concluded that growth, as well as uptake of Fe and Ca were enhanced by carbon nanotubes, likely due to their effects on water uptake. However, that study did not include any other forms of C addition as controls, and so it may just be an effect of added carbon, not because it was in the form of nanotubes.

CNTs in the natural environment will eventually end up in soils (Nowack and Bucheli 2007) and waters where plants and other organisms will be exposed to them. The proportion of bulk carbon particle content in soils can impact the mobility of molecules in the soil and CNTs have been shown to have significant effects on biogeochemical cycling of nutrients and the translocation of metals into the plant system (Rodrigues et al. 2013). As CNTs accumulate in biological systems, they can also block the flow of nutrients and other materials within plants (Nowack and Bucheli 2007). Other studies have shown uptake of salts can be inhibited by the

presence of CNTs (Yang et al. 2013). Therefore, presence of CNTs in the growth medium of plants may alter the availability/mobility of nutrients in solution which may affect the uptake and accumulation of nutrients within plants.

3.6. Conclusions

This research aimed to investigate the hypotheses:

1) CNTs inhibit plant growth when compared to plants treated with AC or No carbon treatment. The results of this experiment showed that root and shoot biomass were significantly lower compared to AC treatments, confirming plants respond differently to addition of insoluble, suspended carbon to nutrient solution in different forms, and that CNTs may inhibit plant growth when compared to the other carbon treatments.

2) CNTs will interfere with uptake and/or accumulation of nutrients in plant tissue. The root tissue of plants treated with AC had higher concentrations of potassium and sulphur than plants treated with industrial or pure CNTs, demonstrating the CNTs show different uptake patterns than the bulk carbon (AC). The shoots had significantly higher concentrations of boron in plants treated with CNTs, but lower concentrations of calcium and sodium in plants that received treatments containing AC. there seemed to be no effect when compared to the No carbon treatment treatments, but these results confirm the hypothesis that CNTs may alter nutrient uptake compared to the AC treatments.

3) There is a stronger effect of CNTs on plant biomass and nutrient uptake with more concentrated carbon treatments. An effect of exposure to different concentrations was only observed through a significant C-type X Concentration interaction for Cr in plants. For biomass and the other elements such an effect was not observed. However, one reason may be that the range of concentrations was not wide enough to see effects. On the other hand, very high

concentrations, such as 1000 mg L^{-1} used in other studies are not relevant to the situation in the field. It therefore appears that at least in the range of exposure used in this experiment is not an important factor.

The rice plants used in this study showed much variation in growth, which may have affected the outcomes of this study. However, the results certainly indicate that exposure to carbon nanotubes may affect growth and nutrient uptake in rice. This therefore warrants further research because CNTs are in development for many applications, including for uses in the agricultural industries such as enhanced fertilizers, herbicides, and pesticides, it is important to understand the interactions that may occur when plants, especially important crop species like rice, are exposed to CNTs.

4. DISCUSSION

4.1. Biomass

In the present study, we observed increased biomass in spring wheat plants exposed to high concentrations of zinc oxide nanoparticles at the roots. However, the same effect on plant biomass was not observed in durum wheat plants exposed at the roots, or in either species when the zinc oxide nanoparticles were applied as a foliar spray. Sheoran et al. (2021) also observed increased biomass in wheat plants exposed to zinc oxide nanoparticles at the roots. Studies have shown similar results for other plant species including peanut (Prasad et al. 2012), *Arabidopsis* (Wang et al. 2016), carrot (Elizabeth et al. 2017), and rice (Zhang et al. 2019). This indicates that zinc oxide nanoparticles may have a stronger effect when applied to the roots. However, we cannot make any firm conclusions regarding this comparison because the results were observed in two separate experiments with varying growth conditions. The larger surface area of zinc oxide nanoparticles increases the ionization rate of zinc oxide nanoparticles which results in more available zinc for uptake by the plant (Mortvedt 1992) and contributes to biomass accumulation (Singh et al. 2018). This likely explains the increased biomass observed in wheat plants exposed to zinc oxide nanoparticles.

We observed significantly lower biomass in rice plants treated with CNTs versus those treated with AC or no-carbon treatments. Typically, increased carbon content within growth substrate is expected to positively affect plant growth (Kumar et al. 2021). Higher carbon content in soils has also been shown to stabilize crop productivity (Pan and Pan 2009). However, CNTs have been found to penetrate cell walls and cause damage to plants which results in lowered biomass (Begum et al. 2012, Yuan et. al 2011). This may be the cause for lowered biomass in plants that were treated with the CNTs. A study by Hao et al. (2016) also

demonstrated that CNTs can cause decreased nitrogen assimilation in rice, which inhibited plant growth.

4.2. Element Uptake

We found that ZnO nanoparticles can affect nutrient uptake and accumulation in wheat. The cause may be a result of altered physiological processes within the plant or could also be the result of interactions between nanoparticles and nutrients in the rhizosphere. Zinc oxide nanoparticles have increased surface area and the dissolution rate of zinc oxide in solution is much higher, which typically results in more available zinc ions for uptake by the plant. The increased availability of zinc oxide nanoparticles results in increased plant growth. The uptake and accumulation of many other nutrients can also be affected by the increased biomass and also by the altered interactions with nanoparticle zinc oxide.

Rice plants showed changes in uptake of some elements in response to differences in amounts and types of carbon in the growth medium which suggests that CNTs affected the roots differently than AC, where growth and nutrient accumulation was inhibited. Most notably, CNTs appeared to have significantly different effects on plant growth and nutrient uptake when compared to activated carbon. As CNTs accumulate in biological systems, they can also block the flow of nutrients and other materials within plants (Nowack and Bucheli 2007). Other studies have shown uptake of salts can be inhibited by the presence of CNTs (Yang et al. 2013). Therefore, presence of CNTs in the growth medium of plants may alter the availability/mobility of nutrients in solution which may affect the uptake and accumulation of nutrients within plants. CNTs in the environment will eventually end up in soils (Nowack and Bucheli 2007) and waters where plants and other organisms will be exposed to them. The proportion of bulk carbon particle content in soils can impact the mobility of molecules in the soil and CNTs have been

shown to have significant effects on biogeochemical cycling of nutrients and the translocation of metals into the plant system (Rodrigues et al. 2013).

5. CONCLUSIONS

This research aimed to assess the effects of ENMs on the plant growth and element uptake in important crop species. The results of this study indicate that zinc oxide nanoparticles can affect the biomass and nutrient uptake/accumulation in wheat plants. Similarly, we observed that CNTs can significantly affect biomass and nutrient uptake/accumulation in rice plants. These results show that ENMs interact with plant systems in different ways than their bulk counterparts. The effects of CNTs and ZnO nanoparticles observed in this study are comparable with the findings of other studies. Both CNT and ZnO can have varied effects on plant growth and nutrient uptake. Exposures at high concentrations of either nanoparticle can result in toxicity effects or inhibited growth. Further research is needed to understand the interactions more fully between ENMS and crops plants under various growth conditions. Additionally, the development of CNTs and ZnO nanoparticles for use in agricultural applications should take into consideration not only the potential benefits, but also the potential negative effects of CNTs and ZnO nanoparticles in plant systems.

At the time of experimental design for this project, studies examining the effects of nanoparticles on plants were mostly limited to short term experiments examining effects on germination or the first stages of plant growth. Long-term studies which take place over the entire growth cycle of a plant species will provide more complete knowledge base for the interactions of ENMs in plant systems. Additional studies extending throughout multiple generations, where seeds are harvested from plants treated with ENMs would add useful context for the long-term effects of ENMs in plant systems.

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