2014-01-01

# Impact Of Zinc Oxide Nanoparticles On Green Pea Plant & Seed Quality And Effects On Physiological Traits Of Green Peas, Corn, And Zucchini By Silver Nanoparticles

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# Dedication

To my parents and teachers for their love, support, and encouragement.

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by

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DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Environmental Science and Engineering

THE UNIVERSITY OF TEXAS AT EL PASO

August 2014

## Acknowledgements

I would like to express my heartfelt gratitude to my mentor, Prof. Jorge L. Gardea-Torresdey, for his guidance and encouragement. Thanks for giving me the honour to work with one of the best research groups in the world in the field of environmental chemistry. Your help and support came at the right time in my career when I needed it the most. Thanks for teaching me how to formulate and pursue projects, write manuscripts, review journal articles. You have taught me how to think indepently and probably, the most importantly, how to be humble, stay hungry for success, and mentain a balance between my personal and professional life. Thanks for being supportive, encouraging, and believing in my abilities. I am grateful to you for letting me flourish under your supervision. Working with you was always fun and challeging, which I enjoyed throughout the entire course of time. I am honoured to be a part of your outstading research group. All my future success will be dedicated to you.

I would like to thank Dr. Jose R. Peralta-Videa for your kind attention and suggestions towards improving my manuscripts. Thanks for teaching me the basics of scientific writing. Your training is one of the major factors for making me an indepndent writer. Thanks for being patient and answering all my scientific enquries. I am greatful to you for your advice, support, cooperation, and guidance.

I would also like to extend my thanks to Dr. Jason C. White for serving my committee and for all your valuable suggestions and critiques for my doctoral research. Thanks for honoring me with the post-doctoral scientist position in your group. I appreciate it.

I am thankful to my committee members Dr. Geoffrey B. Saupe and Dr. John Walton for serving my committee and insiteful critiques.

My completion of the projects could not have been accomplished without the support of Dr. Gardea's group members. Thank you all for your collaboration and cooperation. A special thanks to Dr. Susmita Bandyopadhyay, Cyren Rico, Dr. Lijuan Zhao, Dr. Jose Angel Hernandez-Viezcas, Erving Morelius, and Carlos Tamez for your active collaboration and making "my projects" to "our projects".

I would also like to thank the Environmental Science and Engineering Program of UTEP for accepting me and giving a chance to pursue my PhD under Prof. Gardea's supervision. A special thanks to the Chemistry Department for allowing me to use necessary resources.

I wish to thank my parents for their support and encouragement throughout my life. Thanks for being such a amazing teachers. I am also grateful to my in-laws for their love and support.

My deepest gratitute to (my friend) my brother Dr. Rajib Paul and his family for your love and care for us.

My sincere appreciation goes to my affectionate and tallented wife and collaborator, Dr. Susmita Bandyopadhyay. Without your cooperation and encouragement nothing could have been possible. Thanks for helping and guiding me in every step of my life.

Last but not the least, a special thanks to my son, Ashmit Mukherjee, for being such a nice kid (most of the time) throughout last four years, especially in last summer during my proposal defense. Without your cooperation I could have not reached this point of my carrier.

# Abstract

The production and use of numerous engineered nanomaterials (ENMs) have increased exponentially over the past decade. Nanoparticles (NPs), ENMs possessing diameter between 1-100 nm, (NPs), are widely used in many applications. Worldwide consumption of NPs has increased their possible release into the environment. This, in turn, has elevated the extent of the potential impacts of NP exposure to living and non-living organisms. This is why the assessment of the impact of NPs on different environmental components, especially on plants, the producer in the food web, has become a very important aspect of nano-ecotoxicology. However, studies focusing on phytotoxicity and effects on plant life-cycle are very limited. To evaluate the long and short-term phyto-toxicological effects of NPs, we have chosen green peas (*Pisum sativum* L.), corn (*Zea mays*), and zucchini (*Cucurbita pepo*) as the test plants for their worldwide consumption. Different zinc oxide [bare (bare-ZnO), alumina doped ($Al_2O_3$@ZnO), iron doped (Fe@ZnO), and KH550 coated (ZnO@KH550)] and silver [(AgNP and PVP coated (Ag@PVP)] NPs have been chosen due to their enormous global consumption in different industries, e.g., paint, cosmetics, and drug, among others. This project was completed in four phases. In Phase I, the green pea plants (*P. sativum* L.) were exposed to 0, 125, 250, and 500 mg $kg^{-1}$ of 10 nm bare ZnO NPs and bulk ZnO for 25 days in organic matter enriched soil (native soil: potting soil= 1:1) in a growth chamber. Toxicological effects were investigated in terms of plant growth, chlorophyll production, zinc accumulation in different tissues, reactive oxygen species/ROS ($H_2O_2$) generation, stress enzyme activity (catalase/CAT and ascorbate peroxidase/APOX), and lipid peroxidation. Root elongation reduction (48-52%) was observed in all ZnO NP concentrations ($p \leq 0.05$); however, stem lengths were unaffected compared to control. Chlorophyll in leaves decreased, compared to the control, by ~61%, 67%, and 77% in plants

treated with 125, 250, and 500 mg $kg^{-1}$ ZnO NPs, respectively. Bulk ZnO treatments also showed similar results. In roots and leaves, APOX activity decreased in both nano and bulk treatments. However, in leaves, CAT activity decreased in NP treatments but remained unaltered with addition of bulk ZnO. In leaves, there was a 61% increase in $H_2O_2$ production with a twofold increase in lipid peroxidation. From this study, it may be concluded that the nano form of ZnO is more toxic than the bulk form under the growth conditions of this study. Phase II was designed to evaluate the toxicological effects of 10% Fe@ZnO NPs on green peas at 0, 125, 250, and 500 mg $kg^{-1}$ concentrations for 25 days in similar soil type and similar growth conditions. Results were compared with that of Phase I. At 500 mg $kg^{-1}$, zinc bioaccumulation was increased in both root (200%) and stem (31-48%), compared to control, without affecting the iron uptake ($p \leq 0.05$). Chlorophyll content and $H_2O_2$ production decreased by 27% and ~50%, respectively ($p<0.05$), compared to control. Fe@ZnO showed less toxicity than that of bare-ZnO NPs under the applied growth conditions as indicated by zinc bioaccumulation, chlorophyll production, and $H_2O_2$ production. Therefore, iron doping can be considered as a safer approach to reduce toxicity of ZnO NPs in terrestrial plants. Phase III was focused on phyto-toxicological studies of bare-ZnO NPs, alumina@ZnO NPs, and ZnO@KH550 NPs on green pea plant, its life-cycle, and seeds. The plants were grown in a greenhouse with continuous supply of nutrients (fertilizer) in the similar 1:1 organic matter enriched soil for 65 days. Upon harvest, different physiological and biochemical parameters, e.g., fresh and dry weights, leaf chlorophyll *a*, *b*, leaf carotenoids, zinc bioaccumulation, protein and carbohydrate profiles were measured in different parts of the plant, as applicable. No change in plant fresh and dry weights with treatments were observed, except with ZnO@KH550 at 1000 mg $kg^{-1}$ treatment, which showed about one fold (95%) increase in plant fresh weight compared to control. Plant roots showed a significant increase in

Zn accumulation of 5.7x, 5.7x, and 8x treated with 250 mg $kg^{-1}$ bulk ZnO, bare ZnO NP, and $Al_2O_3$@ZnO NP respectively, compared to controls. Similarly, at 1000 mg $kg^{-1}$, bare ZnO NP and $Al_2O_3$@ZnO NP treatments showed significant increases in zinc uptake up to 16x and 36x times compared to controls. Green pea stems showed higher level of Zn accumulation, except with the ionic zinc treatment. The Zn accumulation was in this order: [at 250 mg $kg^{-1}$: bulk (5x), bare (7x), doped (4.7x) and coted (7x); at 1000 mg $kg^{-1}$: bulk (9x), bare (11x), doped (20x) and coted (9x)] compared to control. In leaves, all the treatments (bulk and coated) showed significant increase in zinc uptake (4.6x to 5.3x) except at 250 mg $kg^{-1}$ and 500 mg $kg^{-1}$ treatments. The1000 mg $kg^{-1}$ treatments (bulk, bare, and doped) also showed significant increase in zinc uptake (5.5x to 11x) except for coated and ionic treatments. The aluminum and silicon uptake did not change with one exception at 1000 mg $kg^{-1}$. Amount of chlorophyll-*a* (Chl-*a*) was significantly increased at 250 mg $kg^{-1}$ alimina doped treatment (4.5x) and in all the treatments at 1000 mg $kg^{-1}$ [bulk (3.2x), bare (2.7x), doped (3.6x), coted (2.5x), and ionic (2.4x)] compared to control. However, there was no difference in the amount of chlorophyll-*b* (Chl-*b*) was observed. The total carotenoid was increased significantly at 250 mg $kg^{-1}$ to 10x in doped and 7x times in ionic treatment. The increase was 7.6x in bulk and 8.6x in case of doped NPs at 1000 mg $kg^{-1}$ treatments. The NP treatments also altered seed quality of the pea. The pod lengths, pod weights, and number of seeds per pod did not change among treatments with the exception of alumina doped 250 mg $kg^{-1}$ treatment where the number of seeds per pod decreased by 33% compared to that of bare ZnO NP treatment. In seed (pea), zinc accumulation at 1000 mg $kg^{-1}$ was increased in all the treatments ranging from1.8x to 2.5x, compared to control, except for the ionic treatment. A threefold (3x) increase in aluminum, silicon, and iron content was recorded in all treatments, except with the 250 and 1000 mg $kg^{-1}$ coated treatment. However, copper,

magnesium, phosphorus (except 1000 mg $kg^{-1}$ coated treatment increased 35%), manganese (except 1000 mg $kg^{-1}$ coated treatment increased 2x), potassium bioaccumulation did not change with changing treatments. In carbohydrate profile, formation of non-reducing sugar (sucrose) was increased nearly two folds (1.8x) at 1000 mg $kg^{-1}$ doped treatment, compared to control. The amount of total sugar, starch, reducing sugar, and protein profile remain unaltered. Considering our Phase III results, the $Al_2O_2$@ZnO NP treatments was found to be more toxic to green pea compared to all other different NP treatments. The comparative phyto-toxicity of different AgNPs on monocot (corn) and two dicot (green peas, zucchini) plants were studied in Phase IV. Plants were treated with bare silver NPs (Ag NPs, 20 nm), 0.2 weight percent PVP coated AgNPs (Ag-PVP-L with 30-50 nm and Ag-PVP-S with 20 nm diameters), bulk silver, and silver sulfate (Ag-ions) at 500, 1000, and 2000 mg $kg^{-1}$treatments [ionic treatments were set at 5(Ion-5), 10 (Ion-10), and 20 (Ion-20) mg $kg^{-1}$]. The experiments were done in small glass jars with 50 g soil, 20 ml vermiculite, and 20 ml 25% Hoagland solution for 20 days. Seeds were germinated in a non-contaminated environment (in vermiculite) and then transferred in the test media. In nano-Ag at 1000 mg $kg^{-1}$ and in all 2000 mg $kg^{-1}$ treatments, the fresh weight (FW) was reduced, except with the ionic one. However, the dry weight (DW) remained unaffected in all the treatments. In roots, silver uptake increased in a concentration dependent manner in all the treatments (except the ionic treatment) compared to control. At 2000 mg $kg^{-1}$, all the treatments (except the ionic) increased shoot silver, compared to control. Chlorophyll-*a* increased in Ag-PVP-L treatments at 500 and 2000 mg $kg^{-1}$ treatments. The amount of carotenoid decreased in 500 and 1000 AgNP mg $kg^{-1}$ and same trend was observed in Ag-PVP-S at 2000 mg $kg^{-1}$) treatments, compared to control. In zucchini, dry weight decreased in all the NP treatments except with 500 mg $kg^{-1}$ Ag-PVP-S and AgNP 2000 mg $kg^{-1}$, compared to control. However, the

dry weight decreased in all the NP treatments at all concentrations. At 500 and 1000 mg $kg^{-1}$, root uptake of Ag increased in a concentration dependent manner. On the other hand, only at 500 mg $kg^{-1}$ Ag-PVP-L treatment showed an increase in the shoot silver. Ag-PVP-S treatment increased chlorophyll by 2.25x and carotenoid by 2.6x compared to control. In corn, the fresh and dry weights were not affected by any of the treatments. Root uptake of silver was increased (by15x to 26x) with silver treatments. However, the shoot uptake increased only with Ion-10 treatment. Chlorophyll and carotenoid amounts were not affected by any of the treatments (except 1000 and 2000 mg $kg^{-1}$ Ag-PVP-S treatment). These comparative phyto-toxicological studies of bare, coated, and doped NPs on different higher plants may help to shine light on the mechanism of zinc and NP toxicity.

# Table of Contents

# List of Tables

# List of Figures

# Chapter 1

## Introduction

Nanoparticles (NPs) are natural or anthropogenic materials with at least two dimensions between 1-100 nm (*1*). Due to their very high surface to volume ratio, NPs possess numerous unique properties, e.g., high reactivity, unique optical properties, and bio-compatibility, among others (*2-6*). These properties make them suitable for various uses in different industries, e.g., paint, healthcare, electronics, and catalysis, among others (*7-9*). Worldwide production and widespread use are two of the major sources of NPs in the environment (*10*). Nanoparticles can be divided into two main classes, organic and inorganic (*11*). Organic NPs are of two main types, fullerenes and carbon nanotubes (*11*). Inorganic NPs can be divided into three large groups, namely, metal oxides, metals, and quantum dots (*11*). Inorganic NPs, e.g., zinc oxide (ZnO) NPs, titanium dioxide ($TiO_2$) NPs, silver (Ag) NPs, cerium oxide ($CeO_2$) NPs, are among the most widely used NPs (*12*). Among all the NPs, silver and zinc based NPs are two of the most commonly used NPs in the world. ZnO NPs are being used in different industries, e.g., paint, consumer products; and Ag NPs are used in food, paint, textile industries (*5, 7-9*). Apart from their beneficial effects, these NPs could potentially lead to adverse health and environmental effects (*5-6*). Literature shows that there are numerous reports of NP toxicity on microorganisms, animals, and plants (*13-17*). Nevertheless, the understanding of the mechanism of toxicity is still in its infancy. Investigation of the effects of different NPs on plants, the producer of the food web, is of great importance. There are few reports that illustrate the phyto-toxicological effects of NPs on food plants, e.g., alfalfa (*Medicago sativa*), cilantro (*Coriandrum sativum* L.), tomato (*Solanum lycopersicum* L.), corn (Zea mays), radish (*Raphanus sativus*), rape (*Brassica napus*), cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*), ryegrass (*Lolium perenne*), among others (18-21).

For example, Lin and Zing reported root elongation with ZnO NP treatment on radish (*Raphanus sativus*), ryegrass (*Lolium perenne*), and rape (*Brassica napus*) (*21*). Phyto-toxicity of ZnO NPs was imposed by the disruption in water/nutrient pathways (*21*). Moreover, genotoxic effect of ZnO NPs on soybean (*Glycine max*) was reported by Lopez-Moreno et al. (*22*). On the other hand, there are very few reports of phyto-toxicological studies on algae (*23-25*), and terrestrial plants (*26-29*). Barrena et al. reported that Ag NPs (29 nm) showed reduction in germination in cucumber and lettuce (*26*). Reduction in biomass in *Cucurbita pepo* by AgNPs (< 100 nm) was reported by Stampoulis et al. (*27*). Kumari et al. investigated the cyto-toxicological effects of Ag NPs on *Allium cepa*, which include distributed metaphase, and stickiness, among others (*28*). Yin et al. observed a concentration dependent increase in silver content in common grass (*Lolium multiflorum*) (*29*).

To the best of author's knowledge, there are very few reports on comparative toxicity of coated and doped NPs in higher terrestrial plants. Moreover, there is no report of comparative study of the effects of different NPs (bare, doped, and coated) on seed quality. Organic and inorganic coatings and metal/metal oxide doping may significantly change the surface properties of the NPs. Surface or lattice modified NPs interact differently than the bare/pristine NPs with the environment. Coating and doping something may cause reduction in dissolution and hence affecting the potential of toxicity of a NP. For example, lower toxicity of iron doped ZnO NPs (Fe@ZnO NPs) than undoped ZnO NPs on zebrafish embryos has been reported by Xia et al. (*30*). Lower toxicity was attributed to the lower dissolution of Fe@ ZnO NPs compared to that of the bare/undoped ZnO NPs (*30*). On the other hand, no correlation between the IC 50 values and percentage of iron doping of Fe@ZnO in *Escherichia coli, Pseudomonas putida,* and *Bacillus*

*subtilis* in aquatic media was reported by Li et al. (*31*). Authors have found to have no effect on Fe@ZnO NP dissolution and bacterial toxicity (*31*).

This study was aimed at identifying the comparative toxicological effects of ZnO NPs (bare, doped, and coated) and AgNPs (bare and coated). The effects of different ZnO NPs on seed quality of green pea were also evaluated. The entire study was performed in four phases. Phase 1 was aimed to determine the impact of ZnO NPs on seed germination, plant growth, chlorophyll production, Zn accumulation, and other metabolic processes in green peas grown in soil in a growth chamber, treated with 0-500 mg/kg ZnO NPs. The production of ROS, measured in terms of $H_2O_2$, the lipid peroxidation (LPOX) and the activity of antioxidant enzymes (CAT and APOX) were quantified. In addition, the accumulation of Zn in plant tissues was determined by using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). Bulk ZnO treatments were set to study the growth and physiological changes produced in green pea plants grown in soil impacted with ZnO NPs.

Phase II was aimed to determine the effect of Fe@ZnO NPs on green peas. Plants were grown for 25 days in a growth chamber with 0-500 mg/kg NP in similar 1:1 soil. Toxicological effects were measured in terms of plant growth, chlorophyll concentration, ROS production, activity of anti-oxidative enzymes, e.g., catalase (CAT) and ascorbate peroxidase (APOX).

Phase III determined the comparative toxicological effects on green peas grown in a green house, treated with bare (10 nm), bulk, 2 wt % alumina doped (Al@ZnO, 15 nm), 1 wt % KH550 coated (KH550@ZnO, 20 nm) at 250 and 1000 mg/kg in 1:1 soil with ionic treatments (5 and 20 mg/kg). Early rise green peas (life cycle of 65 days) sowed in black plastic container (Ns-400; diameter: 20cm; tall: 12.5 cm; volume: 3.925 L; Nursery Supplies, Inc.) with 1:1 ratio of local regular soil and Miracle Gro® Potting Mix (Tierra para macetas; Marysville, OH). 200 ml

nutrient solution from injector was added every day [0.72 $g \cdot L^{-1}$ 15 N- 2.2 P- 12.5 K (Peters 15-5-15) to tap water (Control, EC = 1.80 dS/m; pH= 6.62)]. The daily light integral (photosynthetically active radiation) was 15.3 ± 3.1 $mol \cdot m^{-2} \cdot d^{-1}$. The temperatures in the greenhouse were maintained at 26.9 ± 8.6 °C (mean ± standard deviation) during the day and 13.7 ± 4.3 °C at night. The relative humidity was 41.6 ± 19.1%. Effects of different NPs were evaluated by measuring metal uptake, chlorophyll (a and b) concentration, and amount of total carotenoid in leaves. Results showed that in spite of larger size (15 nm) of the doped ZnO NPs, it is exerting more effects than that of bare ZnO NPs (10 nm). This study might have potential to illustrate the effects of crystal and/or surface modification of ZnO NPs on higher terrestrial plants, like green peas.

Phase IV was to perform a comparative toxicological study of different Ag NPs, e.g., bare Ag NPs (20 nm), PVP coated large Ag NPs (Ag-PVP-L, 30-50 nm), PVP coated small Ag NPs (Ag-PVP-Sm, 20 nm), along with bulk silver and silver sulfate as ionic treatment. The main aim of this phase of study was to find out the effects of surface coating and size onto the toxicity of AgNPs on different types of plants (mono- and dicots). The concentration of particles were 500, 1000, and 2000 mg/kg, whereas, the ionic concentration was 5, 10, and 20 mg/kg. Two dicots (green peas and zucchini) and one monocot (corn) were taken as test plants. The results showed that the size and coating on silver significantly change the uptake and Chl-*a*, *b*, and total carotenoid contents in leaves. We observed lower silver uptake in corn and zucchini compared to green peas that might be due to the adverse effects of silver in corn and zucchini roots compared to that of green peas. To investigate this possibility, further investigation is needed.

We reported that toxicological effects and different bare, coated, doped, and Ag (different coated and coated/non-coated) NPs on green peas, corn, and zuccihini and the impact on seed

quality was evaluated.This work may be pioneering towards understanding the mechanism of phyto-toxicity of different NPs in environmental relevant conditions and the longterm impact on seed quality as well as food web.

# Chapter 2

## Physiological effects of nanoparticulate ZnO in green peas (*Pisum sativum* L.) cultivated in soil

(Mukherjee, A.; Peralta-Videa, J. R.; Bandyopadhyay, S.; Rico, C. M.; Zhao, L.; Gardea-Torresdey, J. L.Physiological effects of nanoparticulate ZnO in green peas (*Pisum sativum* L.) cultivated in soil *Metallomics*, **2014**,6, 132-138)- Reproduced by permission of The Royal Society of Chemistry

### Abstract

The toxicity effects of zinc oxide nanoparticles (ZnO NPs) in plants are still largely unknown. In the present study, green pea (*Pisum sativum* L.) plants were treated with 0, 125, 250, and 500 mg/kg of either ZnO NPs or bulk ZnO in organic matter enriched soil and corresponding toxicological effects were measured on the basis of plant growth, chlorophyll production, Zn bioaccumulation, $H_2O_2$ generation, stress enzyme activity, and lipid peroxidation using different cellular, molecular, and biochemical approaches. Compared to control, all ZnO NP concentrations significantly increased ($p \leq 0.05$) root elongation but no effects were observed in stem. Whereas, all bulk ZnO treatments significantly increased both root and stem length. After 25 days, chlorophyll in leaves decreased, compared to control, by ~61%, 67%, and 77% in plants treated with 125, 250, and 500 mg/kg ZnO NPs, respectively. Similar results were found in bulk ZnO treated plants. At all ZnO NP concentrations CAT was significantly reduced in leaves ($p \leq 0.05$), while APOX was reduced in both roots and leaves. In case of bulk ZnO, APOX activity was down regulated in root and leaf and CAT was unaffected. At 500 mg/kg treatment, the $H_2O_2$ in leaves increased by 61% with a twofold lipid peroxidation, which would be a predictive

biomarker of nanotoxicity. This study could be pioneering to evaluate the phytotoxicity of ZnO NPs to green peas and can serve as a good indicator for measuring the effects on ZnO NPs in plants grown in organic matter enriched soil.

**Keywords: E**cotoxicology, Zinc oxide nanoparticle, Bioaccumulation, Reactive oxygen species, Anti-oxidative stress.

## 2.1 Introduction

According to "The Nanotechnology Consumers Products Inventory", ZnO NPs are one of the most widely used nanomaterials.[1] They are extensively used in personal care products, paints, and as anti-microbial agent, among others.[2-4] However, recent literature suggests that NPs produce adverse effects in terrestrial plants. For instance, Lin and Xing reported that ZnO caused phytotoxicity in ryegrass (*Lolium perenne*), radish (*Raphanus sativus*) and rape (*Brassica napus*),[4] displayed as a reduction in root elongation. These researchers concluded that the phytotoxicity of ZnO NPs to ryegrass seedlings was caused by disruption in water- and nutrient pathways.[5] Other negative effects include the reduction of biomass production in wheat (*Triticum aestivum*), reduction in germination and root elongation in some desert plants,[6] and changes in the activities of various soil enzymes such as catalase, protease, and peroxidase.[7] Kim et al. reported that ZnO NPs induced excess Zn bioaccumulation in *Cucumis sativus* grown in soil treated with ZnO NPs,[8] and Lopez-Moreno et al. reported that ZnO NPs are genotoxic to soybean (*Glycine max*).[9]

The risk of soil contamination with ZnO NPs increases with the increased use of these NPs in goods and consumer products. Reports indicate that biosolids from wastewater treatment plants in the United States end in agricultural fields and could be contaminated with metal oxide NPs.[10]

These NPs can produce toxicity to plants, whose mechanisms and consequences are not well understood yet. In the case of ZnO NPs, it seems that the toxicity is due to the release of ionic $Zn^{7, 8}$ and/or by NPs induced oxidative stress.[11-13] In wheat, the phytotoxicity of ZnO NPs was associated to oxidative stress.[13] On the other hand, Zhao et al. reported that ZnO NPs produced toxicity to corn (*Zea mays*) Golden variety, by affecting chlorophyll production when the soil was amended with alginate, a natural organic matter.[14] Concerning the mechanism of toxicity, the most recent literature indicates increased level of intracellular reactive oxygen species (ROS) production.[11-13] ROS molecules can accumulate and cause membrane damage which might lead to apoptotic cell death.[15]

Plants overcome the damage from ROS molecules by increasing the activity of stress enzymes like catalase (CAT) and ascorbate peroxidase (APOX), among others. However, to the best of the authors' knowledge, only two reports have described the effects of ZnO NPs in plants.[14,16] But, the effect of ZnO NPs on green pea (*Pisum sativum* L.) has not been documented in terms of ROS generation and corresponding up and down regulation of antioxidant enzymes. Green peas are one of the widely used legumes in healthy diets because of the high protein content, presence of essential amino acids (lysine and leucine), vitamins and minerals like potassium, phosphorus, calcium, copper, iron, and Zn.[17,18] As this legume is cultivated in a wide range of soils, it could be grown in soils amended with biosolids contaminated with metal oxide NPs. Therefore, there is an urgent need to understand the toxicity of ZnO NPs to important food crops like green peas.

This study was aimed to determine the impact of ZnO NPs on seed germination, plant growth, chlorophyll production, Zn accumulation, and metabolic processes in green peas grown in soil treated with ZnO NPs. The production of ROS, measured in terms of $H_2O_2$, the lipid

peroxidation (LPOX) and the activity of antioxidant enzymes (CAT and APOX) were quantified. In addition, the accumulation of Zn in plant tissues was determined by using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). Bulk ZnO treatments were set to study the growth and physiological changes produced in green pea plants grown in soil impacted with ZnO NPs.

## 2.2 Materials and Methods

### 2.2.1 Characteristics of ZnO NPs and experimental soil

The ZnO NPs (10 nm commercial spheroid, Meliorum Technologies, New York) were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). The ZnO NPs were dispersed in DI water by ultra-sonication. Hydrodynamic diameters and $\zeta$-potential of the ZnO NPs at various concentrations (125, 250, and 500 ppm) were measured by suing Malvern Zetasizer (Nano-ZS 90, Malvern). Bulk ZnO (Sigma-Aldrich) was used at the same concentrations for comparison purposes. The bulk ZnO was previously characterized.[21]

The original soil was collected from Horizon, TX (31°51′59.06″N; top 20 cm), air-dried and sieved through a 2 mm mesh prior to experimental use. The soil type was classified as sandy loam soil (percentages of clay, silt, and sand of 3.73%, 12.15%, and 84.1%, respectively).[14] Due to the low organic matter content of the Horizon soil, the experiments were performed in a 1 : 1 mixture of the native soil with high organic matter soil (Scotts, premium potting soil). The potting soil was purchased from a nursery store to keep appropriate pore size and augment soil fertility. After mixing, the soil properties were: pH 8.45 ± 0.3, CEC 8.02, and Zn concentration 38.08 mg $kg^{-1}$.

### 2.2.2 Soil preparation (Mixing NPs with soil)

Suspensions of bare ZnO NPs were prepared at 0 (control), 125, 250, and 500 mg ZnO NPs/kg of soil by dispersing desired amounts of NPs in Millipore water (MPW) and sonicated for 30 min to avoid aggregation (Crest Ultrasonics, Trenton, NJ. Model 275DA; 120 volt, 3 amp, 50/60 Hz). The pots (20.3 cm × 18.5 cm, Lawn & Garden section; Wal-Mart Inc.) were filled with the soil mixture. Then, the soil was amended with the NP suspensions (well mixed) and kept 24 h for stabilization. Next day, the seeds were planted in the NP amended soil pots. Each treatment, including control (no NPs) was set in four replicates. No additional fertilizer was used.

### 2.2.3 Seed germination and exposure

Seeds were soaked in a 10% sodium hypochlorite solution for 20 min, rinsed three times with MPW and immerse in MPW for 2 h. After that, seven seeds were sown in each pot. The pots were placed in the growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) with 14-h photoperiod, 25/20°C day/night temperature, 65% relative humidity, and 340 μmol $m^{-2}$ $s^{-1}$ light intensity. After 5 d, the numbers of germinated seeds were counted in each pot and the percent germination (%G) rates were calculated using the formula described elsewhere.[6]

### 2.2.4 Zn uptake by green pea plants

To study the Zn uptake from the soil treated with ZnO NPs, green pea seeds were germinated and grown for 25 days in pots filled with the soil mixture (3 kg per pot). The pots were watered for 25 days with deionized water, enough to maintain the soil close to its water holding capacity. After this period of time, plants were harvested for further analysis. Plants were thoroughly washed with tap water for 5 min and rinsed with 2% $HNO_3$ solution, followed

by washing with DI water for three times.[20] For the uptake study, roots, stems, and leaves were separated and dried at 60 °C for 24 h.

The dry samples were digested in a microwave acceleration reaction system (CEM MARSx, Mathews, NC) following the USEPA 3051 method using plasma pure $HNO_3$ and $H_2O_2$ (1:4) [33] and analyzed for Zn concentration by ICP-OES (Optima 4300 DV, Perkin-Elmer, Shelton, CT). Blank, spiked samples, and 1570a standard reference material (spinach leaves) were used to validate the analytical procedure of Zn measurements. Ten blank samples were analyzed to calculate the detection limit of Zn.

### 2.2.5 Chlorophyll estimation (SPAD Measurement)

Leaf greenness (or relative chlorophyll content) of all plants was measured using a hand-held SPAD chlorophyll meter (Minolta Camera Co., Osaka, Japan) after 15 and 25 days of growth.

### 2.2.6 Biochemical assays

#### 2.2.6.1 Hydrogen peroxide analysis

The analysis of $H_2O_2$ content was conducted following the protocol previously published by Gay and Gibicki (2000).[34] Fresh plant tissues (~500 mg) were powdered in liquid nitrogen and homogenized in 4 mL of 100 mM potassium phosphate buffer (pH 6.8). The mixture was diluted to give a final concentration of 25 mM $H_2SO_4$, 100-150 μM xylenol orange, and 100-250 μM ferrous iron (ferrous ammonium sulfates) in a volume of 2 ml. After 30 min incubation in the dark, the absorbance was measured at 560 nm, with XO/$Fe^{2+}$ as blank.

#### 2.2.6.2 Lipid peroxidation analysis

The lipid peroxidation in plants was determined following the method of Heath and Packer[35] and reported as Thiobarbituric Reactive Species (TBARS)[35] with extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$. The plant leaf (300 mg) was homogenized in 2 mL 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged (Eppendorf AG bench centrifuge 5417R, Hamburg, Germany) at 10,000×*g* for 20 min. A 1 mL aliquot of the supernatant was added with 1 mL of TCA (20%) containing 0.5% (w/v) thiobarbituric acid (TBA) and 100 μL butylated hydroxyltoluene (BHT, 4% in ethanol). The mixture was heated at 95°C for 30 min, then quickly cooled on ice and centrifuged at 10,000×*g* for 15 min. The absorbance was measured at 532 nm. The unspecific turbidity of the sample was measured at 600 nm and subtracted with the absorbance at 532 nm.

**2.2.6.3 Enzyme assay**

The enzymes were extracted according to Lee and Lee.[36] Fresh plant samples (~200 mg) were ground in liquid nitrogen and homogenized in cold solution of 2 mL 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamin-tetraacetic acid, 1% (w:v) polyvinylpyrrolidone and 0.5% (v:v) Triton X-100. The homogenate was centrifuged at 18,000×g for 25 min at 4°C. The supernatant was collected and stored at -80°C.

All enzyme activity assays were done at 25°C using 1 mL volume of reaction mixture. Protein content was determined according to the method of Bradford[37] using bovine serum albumin as standard. The enzyme kinetics for the assays was recorded in a Perkin Elmer Lambda 14 UV/Vis Spectrometer (single-beam mode, Perkin-Elmer, Uberlinger, Germany). The catalase (CAT) activity was assayed by monitoring the degradation of $H_2O_2$ (extinction coefficient 39.4 $mM^{-1}$ $cm^{-1}$) at 240 nm.[38] The reaction mixture contained 980μL 10 mM $H_2O_2$ and 20μL crude enzyme extract. One unit of CAT is defined as the amount of enzyme necessary to decompose 1 μmol of $H_2O_2$ per minute. The ascorbate peroxidase (APOX) activity was measured following

the method of Nakano and Asada[39] by monitoring the decrease in AsA (2.8 $mM^{-1}$ $cm^{-1}$) content at 290 nm for 2 min. The reaction mixture consisted of 936μL 50mM phosphate buffer (pH 7.4), 4μL 25 mM ascorbate (AsA), 10μL 17mM $H_2O_2$ and 50μL enzyme extract. One unit of APOX activity is defined as 1 μmol of AsA oxidized per minute.

### 2.2.7 Statistical Analysis

All the treatments were done in four replicates and arranged in a completely random design. Zn concentration in roots, stems, and leaves were reported as averages of four replicates ± standard errors. A one-way ANOVA test was performed followed by Tukey-HSD test using the statistical package SPSS Version 19.0 (SPSS, Chicago, IL). Statistical significance was based on probabilities of $p \leq 0.05$.

## 2.3 Results and Discussion

### 2.3.1. Size and ζ-potential of the ZnO NPs

Variations in size, pH, and ζ-potential of the ZnO NPs at the concentrations used are shown in Table 1. As can be seen in this table, the size of the NPs increased in suspension (DI water) compared to its dry, solid state. As the concentration of NPs increased, the hydrodynamic diameter decreased; however, the differences were not statistically significant.[19] As a result of that, larger nanoparticles formed, which gravitate out of the solution, leaving behind comparatively smaller NP-assemblies. However, no statistically significant changes in ζ-potential and pH were observed.

**Table 2.1** Size, ζ-potential, and pH of ZnO NPs in DI water suspension. Each data is average of three trials. Each measurement was average of 100 readings.

| ZnO NPs (10 nm, in DI water) | Size (nm) | Zeta Potential (mV) | pH |
|---|---|---|---|
| 125 mg/L | 385.2 (±1.61) | 14.5 (±0.8) | 7.92 (±0.006) |

| | | | |
|---|---|---|---|
| 250 mg/L | 326.5 (±7.00) | 17.8 (±0.3) | 7.95 (±0.006) |
| 500 mg/L | 292.9 (±2.70) | 18.5 (±0.5) | 7.98 (±0.005) |

### 2.3.2 Accumulation of Zn in green pea tissues

Zinc concentrations in dry pea plant tissues are shown in Figure 1. As seen in this figure, plants treated with 250 and 500 mg/kg ZnO NPs had nearly 2× and 4 × more Zn in roots compared to 125 mg/kg treatments, respectively. Figure 1B shows that the Zn concentration in roots, from the 250 mg/L bulk ZnO treatment, was similar to the concentration found with 250 mg/L NP treatment. However, the Zn found in roots, from NP at 500 mg/L, was ~2× compared to Zn in roots of plants treated with bulk ZnO at 500 mg/L. A previous study has shown about six and three times increase in Zn uptake by corn roots and shoots, respectively, in plants grown with 800 mg/kg ZnO NP, compared to plants treated with 100 mg/kg.[20]

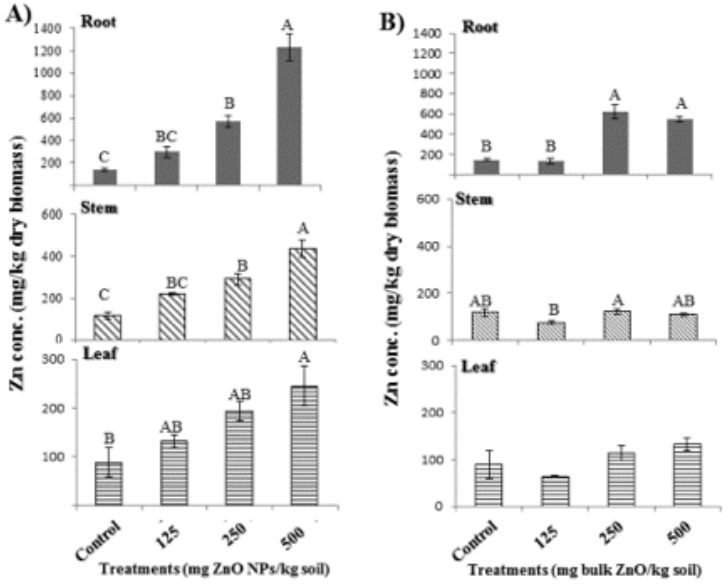


**Fig. 2.1 A**. Zn concentrations in roots, stems, and leaves in green peas grown for 25 days in soil, treated with 0 (control)-500 mg/kg ZnO NPs. **B**. Fe concentrations in roots, stems, and leaves in green peas grown for 25 days in soil, treated with 0 (control)-500 mg/kg bulk ZnO. Error bars

stand for stadard errors. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

In stems, green pea plants treated with ZnO NPs at 500 mg/kg accumulated significantly more Zn compared to plants treated with 250 and 125 mg/kg (Figure 1A). However, at all bulk ZnO concentrations, stems of pea plants had Zn concentrations similar to control stems (Figure 1B). At leaf level, the Zn concentration at the highest ZnO NPs concentration was different only compared to the control. The translocation rates (defined as the ratio of the Zn concentration in shoots to that in roots) for control, 250 mg/kg, and 500 mg/kg ZnO NP treatments were 0.83, 0.51, and 0.35, respectively. On the other hand, stems and leaves of plants treated with bulk ZnO had similar Zn concentrations as control plants (Figure 1B). The higher uptake and translocation in ZnO NP treated plants could be due to the smaller particle size. Hao et al.[21] reported that bulk ZnO has particle of 2000 nm (2920 nm hydrodynamic size), while the ZnO NPs used in our study have primary size of 10 nm and hydrodynamic size of ~385, ~327, and ~293 nm for the 125, 250 and 500 mg/kg treatments, respectively. Previous study showed that the ZnO NPs were taken up by corn roots with a very low translocation to the shoots.[20] In addition, at high concentration, the ZnO NPs could be adsorbed to the root surface avoiding the uptake and translocation because there were no changes in particle and ζ-potential in the NP suspensions.

**2.3.3 Effects of ZnO NPs on plant growth**

The root and stem length of green pea plants grown for 25 days in soil treated with 0-500 mg/kg of ZnO NPs are shown in Figure 2. As can be seen in Figure 2, all ZnO NP/bulk ZnO treatments significantly increased root elongation ($p \leq 0.05$). Root lengths in all treatments were approximately 2× longer than that of control. On the other hand, compared to control, the stem

lengths were increased by approximately 18%, 30%, and 29%, respectively for 125, 250, and 500 mg/kg ZnO NPs (Figure 2). However, the differences were not statistically significant. It is interesting to point out that plants treated with ZnO NPs had significantly larger roots compared to stems. In bulk ZnO treated pants, there were no differences between root and stem lengths (Figure 2B). It is possible that the Zn concentration in stems and leaves of ZnO NP treated plants reached toxic levels that reduced stem lengths. Reports indicate that more than 200 mg/kg of Zn are toxic *Lolium perenne L. cv Apollo* leaves.[22]

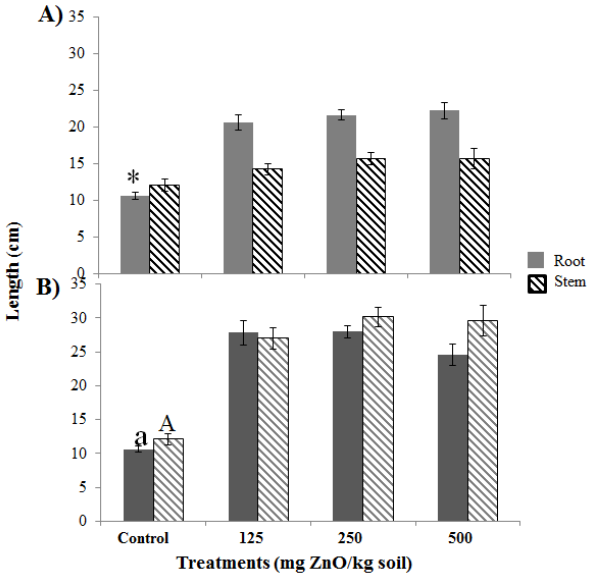


**Fig. 2.2** **A**. Root and stem lengths of green pea plants grown for 25 days in soil, treated with 0(control)-500 mg/kg ZnO NPs. **B**. Root and stem lengths of green pea plants grown for 25 days in soil, treated with 0 (control)-500 mg/kg bulk ZnO. Error bars stand for stadard errors. Bar with the asteric (*) symbol, a, and A show statistically significant differences at $p \leq 0.05$.

These results indicate that at all concentrations tested ZnO NPs promoted the root growth of green pea plants cultivated in organic matter enriched soil. Priester et al.[23] reported that ZnO NPs at 500 mg/kg increased the root biomass in soybean. However, more experiments are

needed to determine the effects of lower ZnO NP concentrations on green pea plants growth. Special attention deserves the study of the effects of ZnO NPs in nodulation of green pea plants, as this is a key factor in legume plants growth.

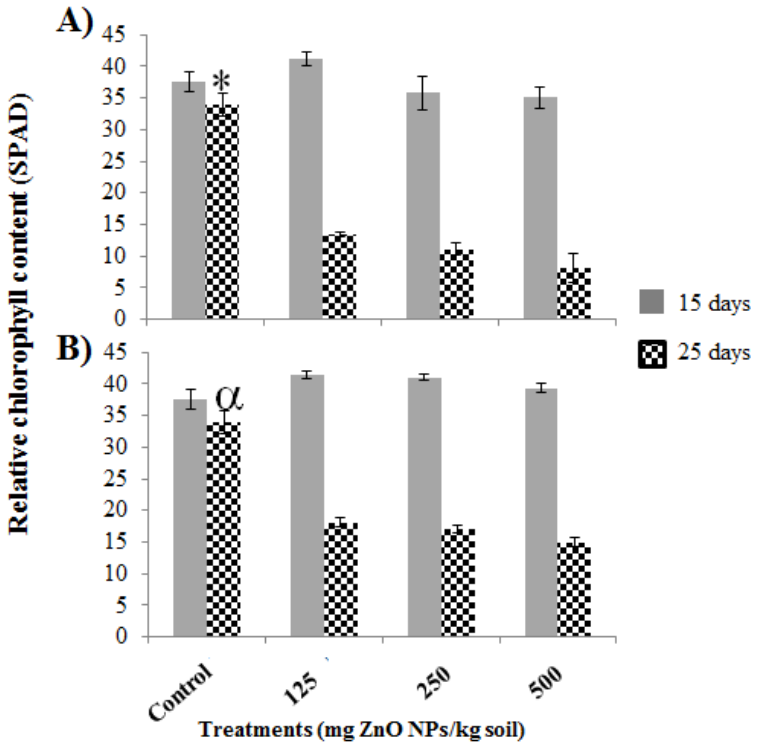


**Fig. 2.3 A**. Chlorophyll contents in the leaves of green pea plants grown for 15 and 25 days in soil, treated with 0(control)-500 mg/kg ZnO NPs. **B**. Chlorophyll contents in the leaves of green pea plants grown for 15 and 25 days in soil, treated with 0(control)-500 mg/kg bulk ZnO. Error bars stand for stadard errors. Bar with the asteric (*) symbol and α show statistically significant differences at $p \leq 0.05$.

As can be seen in Figure 3, the relative chlorophyll content was not impacted by ZnO NPs after 15 days. However, after 25 days, the leaves of ZnO NP treated plants look less green and the SPAD measurements showed a decrease in chlorophyll of ~ 61%, 67%, and 77% at 125, 250, and 500 mg/kg ZnO NPs treatments, respectively. Similar results were found in plants

treated with bulk ZnO (Fig. 3B).This means that Zn started to produce toxicity when the plants were at middle age. It has been reported that the substitution of the central atom of chlorophyll by Zn damaged the mechanism in stressed plants.[24] As stated by Kupper et al.,[24] "This substitution prevents photosynthetic light-harvesting in the affected chlorophyll molecules, resulting in a breakdown of photosynthesis." As can be seen in Figure 1, the Zn concentration in leaves varied from ~150 to 200 mg/kg dry weight biomass and there was an increasing trend with increased NP concentration. In bulk ZnO treated plants, the Zn concentration in leaves varied from 50 to ~150 (Fig. 1B). It is possible that at all the treatments, the Zn concentration in pea leaves had approached the threshold toxicity limit and impacted the chlorophyll synthesis after 25 days.

**2.3.4 $H_2O_2$ generation induced by ZnO NPs**

Previous reports have shown that most abiotic and biotic stress result in an increased production of ROS.[15,25] Inside the cells, there are different types of ROS molecules. In the present study, the ZnO NP concentrations used did not induce overproduction of $H_2O_2$ in green pea roots and stems (Figure 4). However, in leaves, the 500 mg/kg treatment induced overproduction of $H_2O_2$ (61% higher compared to control leaves), which is an indication of oxidative stress. None of the bulk ZnO treatments increased the $H_2O_2$ concentration (Fig. 4B). Perhaps this was due to the lower concentration of Zn found in bulk ZnO treated plants (Fig. 1B). Previous studies reported that zinc induced the production of free radicals in various plants.[26-28]

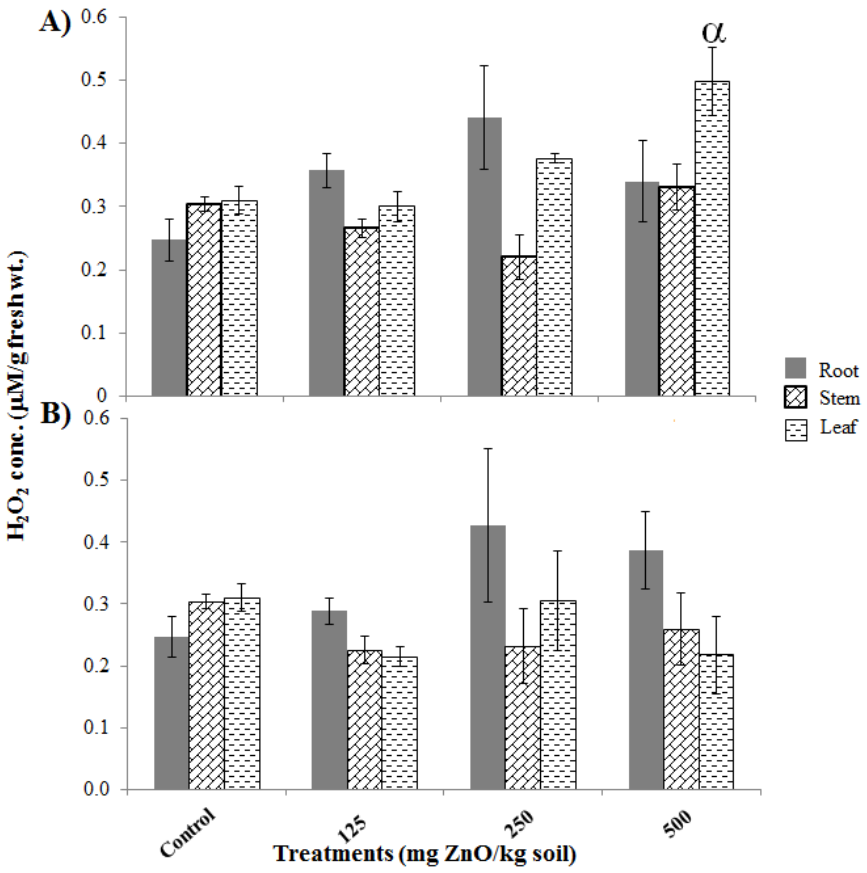


**Fig. 2.4** **A**. $H_2O_2$ concentration in roots, stems, and leaves of green pea plants grown for 25 days in soil, treated with 0 (control)-500 mg/kg ZnO NPs. **B**. $H_2O_2$ concentration in roots, stems, and leaves of green pea plants grown for 25 days in soil, treated with 0 (control)-500 mg/kg bulk ZnO.Error bars stand for stadard errors. Bar with the symbol (α) shows statistically significant difference at $p \leq 0.05$.

### 2.3.5 $H_2O_2$-scavenging enzyme activities

The control of oxidant levels is achieved by antioxidative systems. Enzymatic scavengers of activated oxygen, e.g., APOX and CAT are important component of the defense system in plants.[29] CAT and APOX enzymes are known to be involved in the detoxification of $H_2O_2$ by converting the $H_2O_2$ to water and oxygen.[15] We determined the concentration of both CAT and APOX in different tissues at 25 days. As shown in Figure 5A, none of the ZnO NP treatments

affected the concentration of APOX in stems; however, all the ZnO NP treatments reduced the concentration of APOX in roots (about 2× less), although overproduction of $H_2O_2$ was not observed in roots. In leaves, though overproduction of $H_2O_2$ was observed at 500 mg/kg treatment, the concentration of APOX enzyme was significantly reduced ($p \leq 0.05$). Similar results were found in roots and leaves of bulk ZnO treated plants. In stems, there was a concentration-dependent increase of APOX; however, the differences were not statistically significant (Fig. 5B). CAT activity also was not significantly changed in response to the high level of $H_2O_2$ in root and stem. However, in leaves, all ZnO NP concentrations reduced CAT activity (Fig. 6A). A similar trend was observed in bulk ZnO treated plants but, in this case, the differences were not statistically significant (Fig. 6B).The inhibition of antioxidative enzymes induced by ZnO NPs resulted in the $H_2O_2$ accumulation (statistically significant at 500 mg/kg treatment, Fig. 4A). This confronts the defensive system of the plant. Zhao at al. reported a decrease in APOX and CAT activity in corn plants grown in organic soil treated with 400 mg/kg of ZnO NPs.[14] However, Hernandez-Viezcas et al. reported that in roots of velvet mesquite (*Prosopis juliflora-velutina*) grown in soil treated with ZnO NPs at 4000 mg/kg, there was an increase in CAT activity and a decrease in APOX. However, at 500 mg/kg only APOX showed an increase in mesquite stems.[30] It is important to point out that $H_2O_2$ is just one of the ROS molecules. There are other important ROS molecules in the cells (e.g., superoxide radicals, peroxide radicals, hydroxyl radicals, and singlet oxygen, among others) that could explain the relative discrepancies between the $H_2O_2$ concentration and the $H_2O_2$ scavenging enzyme activities.[31]

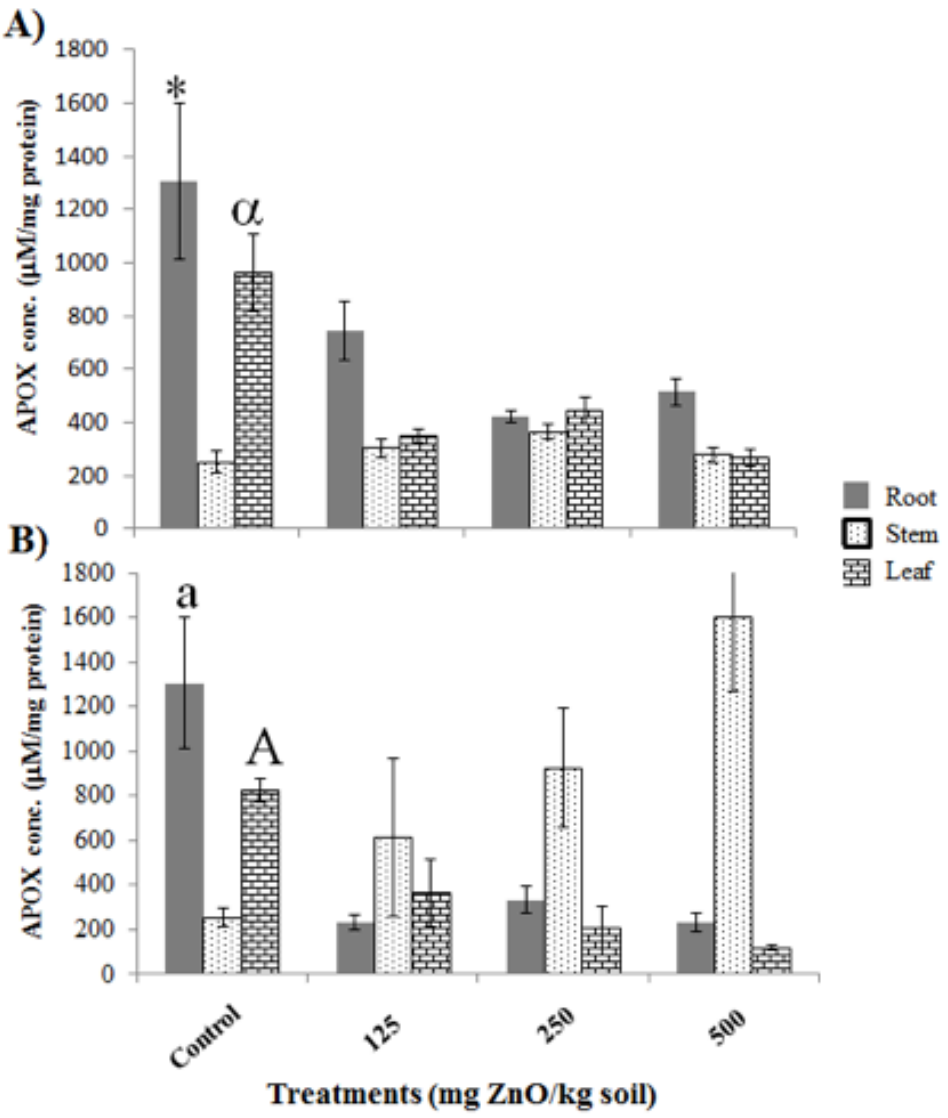


**Fig. 2.5 A**. APOX concentration in roots, stems, and leaves of green pea plants grown for 25 days in soil, treated with 0 (control)-500 mg/kg ZnO NPs. **B**. APOX concentration in roots, stems, and leaves of green pea plants grown for 25 days in soil, treated with 0 (control)-500 mg/kg bulk ZnO. Error bars stand for stadard errors. Bars with the symbols show statistically significant differences at $p \leq 0.05$.

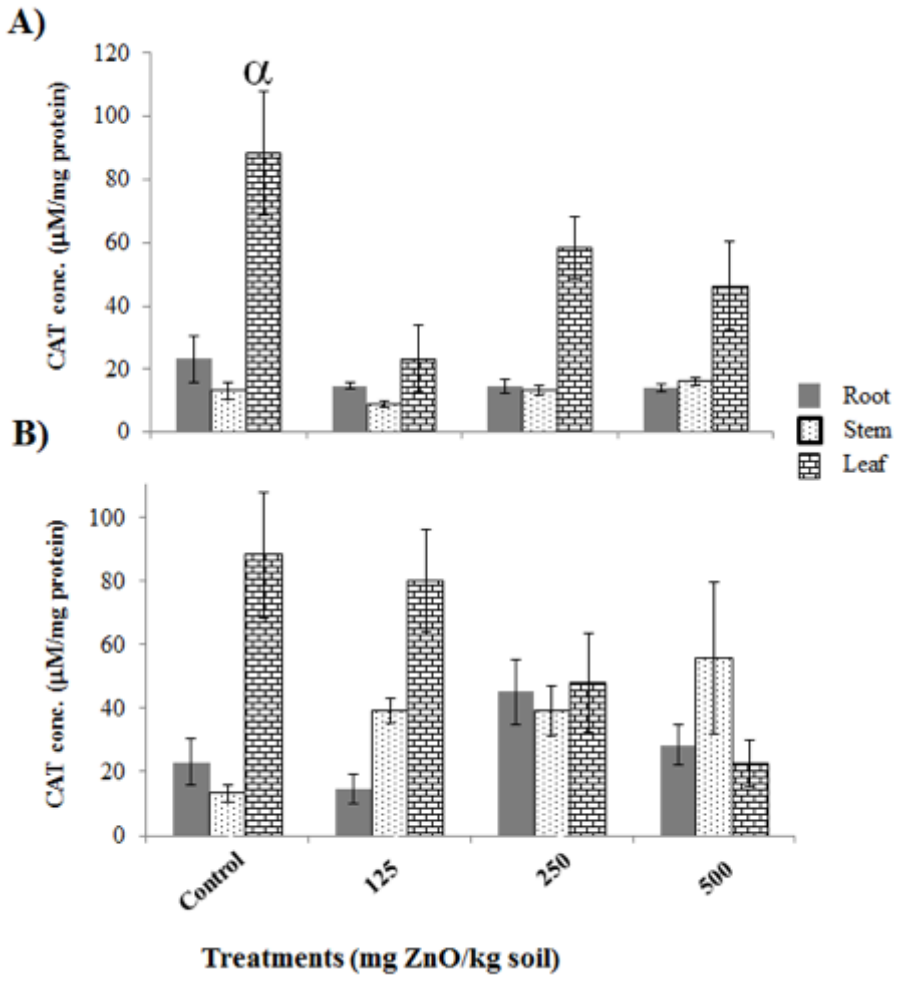


**Fig. 2.6** **A**. CAT concentration in roots, stems, and leaves of green pea plants grown for 25 days in soil, treated with 0 (control)-500 mg/kg ZnO NPs. **B**. CAT concentration in roots, stems, and leaves of green pea plants grown for 25 days in soil, treated with 0 (control)-500 mg/kg bulk ZnO. Error bars stand for stadard errors. Bar with the symbol shows statistically significant difference at $p \leq 0.05$.

### 2.3.6 Lipid peroxidation

Imbalances in the accumulation and removal of $H_2O_2$ and other ROS molecules result in oxidative stress characterized by oxidative damages to proteins, lipids, and DNA.[32] To test if $H_2O_2$ accumulation causes membrane damage and lipid peroxidation in green pea plants, roots, shoots, and leaves were analyzed. TBARS, a byproduct generates from lipid peroxidation (LPOX), was measured. As shown in Figure 7A, the concentration of TBARS in leaves of 500 mg/kg ZnO NPs treated plant was significantly higher than that of control, which concurs with over production of $H_2O_2$ generation in leaves at that NPs concentration. Overproduction of $H_2O_2$ and other ROS molecules such as superoxide radicals, peroxide radicals, hydroxyl radicals, and singlet oxygen, and the inhibition of antioxidant enzyme activity lead to membrane damage caused by ZnO NP treatments. However, none of the bulk ZnO treated plants showed lipid peroxidation (Fig. 7B), which signifies the less toxic effect of bulk ZnO than the ZnO NPs.

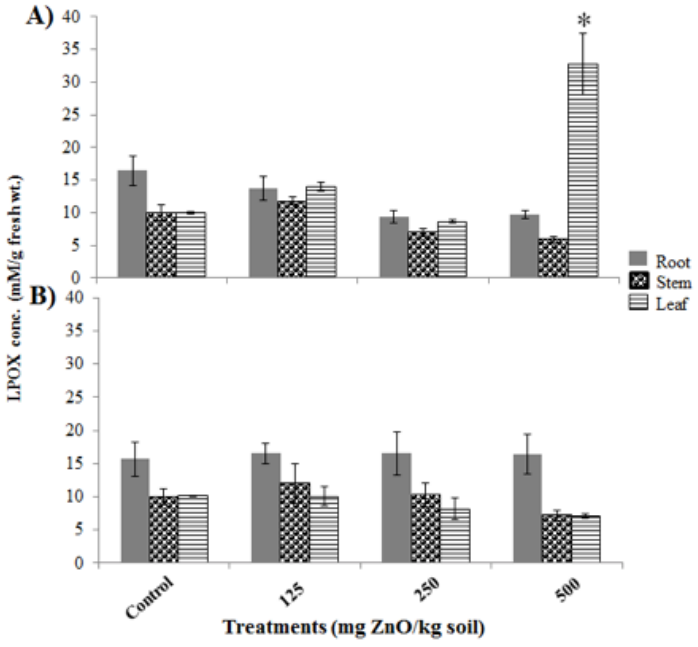


**Fig. 2.7 A**. Lipid peroxidation in roots, stems, and leaves of green pea plants grown for 25 days in soil, treated with 0 (control)-500 mg/kg ZnO NPs. **B**. Lipid peroxidation in roots, stems, and

leaves of green pea plants grown for 25 days in soil, treated with 0 (control)-500 mg/kg bulk ZnO. Error bars stand for stadard errors. Bar with the symbol (∗) shows statistically significant difference at $p \leq 0.05$.

## 2.4 Conclusion

This study has demonstrated that, when grown in organic matter enriched soil treated with ZnO NPs, green pea plants accumulate Zn in roots in a concentration-dependent manner. However, less Zn accumulation was observed in bulk ZnO treated plants. ZnO NPs increased root elongation, whereas, the bulk treatments showed both root and stem elongation. The ICP-OES data showed that this plant is able to translocate the Zn to the aboveground plant parts, in case of NP treatments, but the translocation was insignificant in case of bulk treatments. The high Zn accumulation, mainly in leaves, induces significant accumulation of $H_2O_2$, with a reduction in the activity of the stress enzymes CAT and APOX. But, the bulk treatments showed no effect in $H_2O_2$ and lipid peroxidation. After 25 days, there was a significant reduction in chlorophyll content and increased lipid peroxidation in plants treated with 500 mg/kg of the ZnO NPs. Our results indicate that ZnO NPs induced more toxicity/stress compared to bulk ZnO for green pea plants. Therefore, these results will help to further understand the phytotoxicity of ZnO NPs and possible ecotoxicological impacts in food chain. Future studies must be performed in order to determine the effects of ZnO NPs on the seed quality of pea plants.

# Chapter 3

## A soil mediated phyto-toxicological study of iron doped zinc oxide nanoparticles (Fe@ZnO) in green peas (*Pisum sativum* L.)


### Abstract

Iron doping has shown to reduce toxicity of zinc oxide nanoparticles (ZnO NPs) in several organisms. To the best of authors' knowledge, this is the first report on toxicological studies of Fe@ZnO NPs on terrestrial plant. In this study, green pea plants (*Pisum sativum* L.) were grown for 25 days in soil treated with 10% Fe@ZnO NPs at 0 to 500 mg/kg. Effects were compared with our previous study where phytotoxicity of bare-ZnO NPs had been investigated on green pea plants grown under similar environmental conditions. Different physiological and biochemical growth parameters were measured. Results showed increased Zn bioaccumulation in roots (200%) and stems (31-48%) as the exposed NP concentration increased ($p \leq 0.05$) but Fe absorption was not affected. At 500 mg/kg Fe@ZnO NPs treatment, chlorophyll content (27%) and $H_2O_2$ production (~50%) decreased significantly ($p<0.05$) compared to control. Toxicity of doped ZnO NPs is less than that of bare ZnO NPs as per zinc uptake, chlorophyll content, and ROS ($H_2O_2$) production are considered. Therefore, iron doping can be considered as a safer approach to reduce toxicity of ZnO NPs in terrestrial plants.



**Keywords**: Nanoparticles; Iron doped zinc oxide nanoparticles; phytotoxicity; catalase; ascorbate peroxidase

## 3.1 Introduction

Nanomaterials (NMs) possess at least one dimension less than 100 nm [1, 2] and have enormous surface to volume ratio that is associated with high reactivity, great subcellular transportation, and high bioavailability [3, 4]. These properties open up a wide scope of application of NMs in different industries, e.g., electronics, cosmetics, drug designing, among others [5-8]. However, increasing worldwide production and consumption of NMs leads to unknown hazards to human and environmental health. Therefore, it is of the utmost importance to evaluate the risks associated with NM exposure [9-12]. Zinc oxide nanoparticles (ZnO NPs) are one of the most widely used NPs throughout the world and are often considered as "extremely toxic" in the environment [13, 14]. In plants, released zinc ions from ZnO NPs can substitute the central metal atom of chlorophyll ($Mg^{2+}$), leading to alteration of the photosynthetic core, which in turn causes phytotoxicity [15]. Prior studies have shown that NP treatments can activate ROS production, which causes oxidative stress [2, 16]. In response to the negative impacts of ROS, plants have developed specific defense mechanisms where different anti-oxidative enzymes interact with ROS molecules and transform them into safer by-products [17]. Ascorbate peroxidase (APOX) and catalase (CAT) are two of the most active free-radical scavengers [18] present in plant tissues. These anti-oxidative enzymes deactivate the ROS molecules through conversion to water and oxygen [19].

In recent years, single and multi-element (or corresponding oxide) doping of NPs, addition of foreign atoms in the crystal lattice of NPs, received great deal of attention due to their potential applications. Iron doped zinc oxide NPs (Fe@ZnO) is one of the most important NPs in its class. These NPs have been studied for their conductance [20], ferromagnetic [21], optical [22], and electrical [23] properties. However, very limited research has been reported on their

impacts on living organisms. For instance, George et al. [24] reported that doping of iron in ZnO NPs reduce its toxicity in bronchial epithelial and macrophage cell lines. Xia et al. reported lower toxicity of Fe@ZnO NPs than that of undoped ZnO NPs in zebrafish embryos and lungs of mice [25]. The authors attributed the reduced toxicity of Fe@ZnO NPs to lesser dilution in the growth media [25]. The extent of dissolution decreases (>30% in case of 10% Fe doping) with increase in the amount of Fe compared to that of ZnO NPs [25]. Conversely, Li et al. reported no correlation between the IC 50 (half maximal inhibitory concentration) values and percentage of iron doping of Fe@ZnO in *Bacillus subtilis, Escherichia coli, and Pseudomonas putida* in aquatic media. The authors also mentioned that iron doping did not significantly affect ZnO NPs dissolution or bacterial toxicity [3].

Notably, there is no reported literature about the toxicity of Fe@ZnO NPs on higher plants, which could lead to trophic transfer of NMs into the food web. To the best of authors' knowledge, this is the first report on toxicological study of Fe@ZnO NPs with terrestrial plant grown in environmental relevant conditions. Different biochemical assays were used to measure the effects of Fe@ZnO NPs on reactive oxygen species (ROS) production and the activity of APOX) and catalase (CAT). In addition, inductively coupled plasma-optical emission spectroscopy (ICP-OES) was used to determine the concentration of Zn and Fe in different plant tissues. Phytotoxicity of doped ZnO NPs was compared with our previously reported literature of undoped ZnO NPs and bulk ZnO [26].

### 3.2 Materials and Methods

#### 3.2.1 Synthesis and characterization of 10% Fe@ZnO NPs

The metallorganic precursors, zinc naphthenate (10% of Zn by metal, Strem Chemical, 99.9% pure) and Iron naphthenate (12% Fe by metal, Strem, 99.9% pure) were used for the

synthesis of Fe doped metal oxide nanoparticles. A 50 mL portion of 0.5 M zinc naphthenate was mixed with 6.5 mL of 0.5 M iron naphthenate to make 10% of Fe in ZnO. For flame spray pyrolysis (FSP), the liquid precursor was delivered at the rate of 5 mL/min using a syringe pump atomizing using a two phase nozzle with 5 L/min $O_2$ at a constant pressure drop of 1.5 bar at the nozzle tip. The spray was ignited by a premixed co-delivery of $CH_4$ and $O_2$ (1.5 L/min, 3.2 L/min) forming a spray flame. The ultrafine particles were formed by reaction, nucleation, surface growth, coagulation, and coalescence in the flame environment.

### 3.2.2 Dry characterization

For the X-ray diffraction measurements, the Fe@ZnO NPs were loaded in a Philips PW 1800 diffracting instrument, equipped with Ni-filtered Cu-$K_\alpha$ ($\lambda$=0.154 nm) radiation, ¼° fixed divergence, primary and secondary Soller slit with 0.04 rad aperture and X'Celerator detector. $N_2$ adsorption-desorption measurements were carried out using a Quantachrome NOVA 4000e gas sorption system. The powders were placed in a test cell and allowed to degas for 2 hours at 200°C in vacuum before adsorption measurements. Data were obtained by exposing or removing a known quantity of adsorbing gas in or out of a sample cell maintained at constant liquid nitrogen temperature (77 K). The low resolution TEM, the corresponding selected area electron diffractions (SAED) and High resolution microscopic imaging (HRTEM) of the specimens were investigated with a FEI Titan 80/300 microscope equipped with a Cs corrector for the objective lens. A Fischione high angle annular dark field detector (HAADF), GATAN post-column imaging filter and a cold field emission gun operated at 300kV as an acceleration voltage was used.

### 3.2.3 Soil characteristics

The soil for experiments was collected from Horizon, TX (31°51′59.06″N; top 20 -30 cm). The soil was air-dried and sieved through a 2 mm mesh. The percentages of clay, silt, and sand were 3.65%, 18.15%, and 78.2%, respectively (sandy loam soil). The Horizon soil was high in sand and low in organic matter. To promote better growth condition for the pea plants, the soil was mixed with Miracle-Grow potting mix (1 soil : 2 potting mix, v/v). After complete mixing, the soil had pH of 8.39 ± 0.3, CEC of 8.95, organic matter content 0.16%, 29.16 mg Zn $kg^{-1}$ and 5.1 g Fe $kg^{-1}$ soil.

### 3.2.4 Nanoparticle application to soil

General-purpose garden plastic pots (1L) were filled with soil mixture (300 g each). Then, the soil was spiked with NP suspensions and left for overnight stabilization inside the growth chamber. The as synthesized 10% Fe@ZnO NPs were dispersed in Millipore water (MPW), sonicated ((Crest Ultrasonics, Trenton, NJ. Model 275DA; 120 volt, 3 amp, 50/60 Hz)) for 2 min to minimize aggregation and added to the soil mixture to have 0 (control, no NP), 125, 250, and 500 mg NPs $kg^{-1}$ of soil. Each treatment was replicated four times. No supplementary fertilizer/nutrient was used during the experiments.

### 3.2.5 Seed Germination

Seeds were immersed in 10% sodium hypochlorite solution for 30 min, followed by three times rinsing with MPW and finally, soaked in MPW for 6 h. After that, in each pot, seven seeds were sown at approximately 2.5 cm depth. The pots were placed in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) with 14 h photoperiod, 65± 3 % relative humidity, 25/20 °C day/night temperature, and 340 $\square^{2}\ s^{-1}$ light intensity. The number of germinated seeds was counted after five days and percent germination (%G) was calculated for all the set of experiments [27].

### 3.2.6 Zn and Fe uptake by green pea plants

Seeds were germinated and grown for 25 days in the NP treated soil before harvesting. MPW was used to water the plants each day to maintain steady soil moisture content. Plants were harvested on 26$^{th}$ day for further studies. These were thoroughly washed with tap water for four min and then rinsed with 2% $HNO_3$ solution, followed by rinsing with MPW four times [28]. Roots, stems, and leaves were separated and dried at 60 °C for 2 days prior to use for the determination of Zn and Fe concentrations in different parts of the plants.

Acid digestion (plasma pure $HNO_3$: 30% $H_2O_2$ = 1:4) of dried plant parts were performed in a microwave acceleration reaction system (CEM $Mars_X$, Mathews, NC) following the USEPA 3051 method with slight modification [29]. An ICP-OES (Optima 4300 DV, Perkin-Elmer, Shelton, CT) was employed to determine the concentrations of Zn and Fe in all the samples. Blanks, 1570a standard reference material (spinach leaves), and spiked samples were used to validate the analytical procedure of Zn and Fe measurements. A 5 mg $L^{-1}$ Zn standard was run after every 15 samples to validate the readings. The detection limits for Zn and Fe were established by analyzing five blank samples.

### 3.2.7 Chlorophyll estimation (SPAD Measurement)

After 25 days of growth, the relative chlorophyll contents (leaf greenness) of the leaves were measured using a portable SPAD chlorophyll meter (Minolta Camera Co., Osaka, Japan).

### 3.2.8 Biochemical assays

#### 3.2.8.1 Hydrogen peroxide analysis

Analysis of $H_2O_2$ contents in different parts of the plants was accomplished following Gay and Gibicki's protocol [30]. Fresh plant tissues (~400 mg) were frozen in liquid nitrogen and powdered by using mortar and pestle. Crushed samples were homogenized in 4 ml of 100

mM potassium phosphate buffer (pH 6.8). The mixture was diluted using 25 mM $H_2SO_4$, 100-150 μM xylenol orange (XO), and 100-250 μM ferrous ammonium sulfates up to a volume of 2 ml (1ml reagent+1 ml sample extract). After 30 min incubation in the dark, the absorbance was measured at 560 nm, with XO/$Fe^{2+}$ as blank.

**3.2.8.2. Enzyme assays**

The enzymes were extracted according to Lee and Lee [31]. Fresh plant samples (~200 mg) were frozen and crushed in liquid nitrogen, and homogenized in cold (4 $^{o}$C) solution of 2 mL 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamin-tetraacetic acid, 1% (w:v) polyvinylpyrrolidone, and 0.5% (v:v) Triton X-100. The homogenate was centrifuged at 18,000×g for 30 min at 4°C (Eppendrof centrifuge 5417R, Germany). The supernatant was stored at -80°C.

All enzyme activity assays were performed at room temperature using 1 mL volume of reaction mixture. Protein contents were determined according to the Bradford method using bovine serum albumin as standard [32]. Enzyme kinetics studies were performed using a Perkin Elmer Lambda 14 UV/Vis Spectrometer (single-beam mode, Perkin-Elmer, Uberlinger, Germany).

CAT (EC 1.11.1.6) activity was measured by observing the degradation of $H_2O_2$ at 240 nm (extinction coefficient 39.4 $mM^{-1}cm^{-1}$) for 5 min [33]. The reaction mixture contained 970 μL 10 mM $H_2O_2$, and 30 μL crude enzyme extract (i.e., total volume = 1mL). One unit of CAT is defined as the quantity of enzyme required to decompose 1 μmol of $H_2O_2$ per minute.

APOX (EC1.11.1.11) activity was measured by monitoring the decrease in Ascorbate (AsA) (2.8 $mM^{-1}$ $cm^{-1}$) content at λ=290 nm for 3 min [34]. One unit of APOX activity is defined as 1 μmol

of AsA oxidized per minute. The reaction mixture consisted of 936 μL 50 mM phosphate buffer (pH 7.4), 4 μL 25 mM (AsA), 10μL 17 mM $H_2O_2$ and 50 μL enzyme extract.

### 3.2.9 Statistical Analysis

Results were reported as mean of four replicates ± standard error. SPSS Version 19.0 (SPSS, Chicago, IL) was used to perform one-way ANOVA tests and corresponding Tukey-HSD tests to check the statistical significance at $p \leq 0.05$.

## 3.3 Results and Discussion

### 3.3.1. Size, Surface Area, Crystal Structure, and Z-Potential of Fe@ZnO NPs

Phase analysis, determination of the structural properties and crystallite sizes of pure and Fe@ ZnO NPs were carried out using powder XRD (Fig. 1). The Fe@ZnO NPs had lattice parameters close to the reported values [35]. From the structural model, the apparent crystallite sizes were extracted to determine extra peak width from peak broadening. The apparent crystallite sizes and residuals are listed in Table 1.

**Table 3.1** Particle size evaluation of pure and 10% Fe doped ZnO NPs

| **Sample** | **BET($d_{BET}$) (nm)** | **XRD($d_{XRD}$) (nm)** | **TEM ($d_{TEM}$) (nm)** | **Size in water* (nm)** |
|---|---|---|---|---|
| Pure ZnO | 20.2 | 15.8 | 22 | 130 |
| 10%Fe-ZnO | 8.3 | 5.5 | 10 | 1780 |

### 3.3.2. Specific surface area and microscopic measurements

BET surface area measurement is related to the average equivalent primary particle size as $d_{BET} = 6000/(d \cdot S_A)$ [36], where $d_{BET}$ is the average diameter of the particles, $S_A$ represents the

measured surface area of the powder in $m^2/g$, and *d* is the theoretical density in $g/cm^3$. The specific surface area of pure and Fe@ZnO nanoparticles were 58 (±3) and 80 (±2) $m^2/g$, respectively. From the above relation, the particle sizes were found to be 18.4 nm for the pure ZnO and 13.4 nm for Fe@ZnO. The larger surface areas of Fe@ZnO compared with pure ZnO suggest the reduction of the crystallite size due to Fe incorporation in the lattice. The morphology of the nanoparticles was studied with TEM.

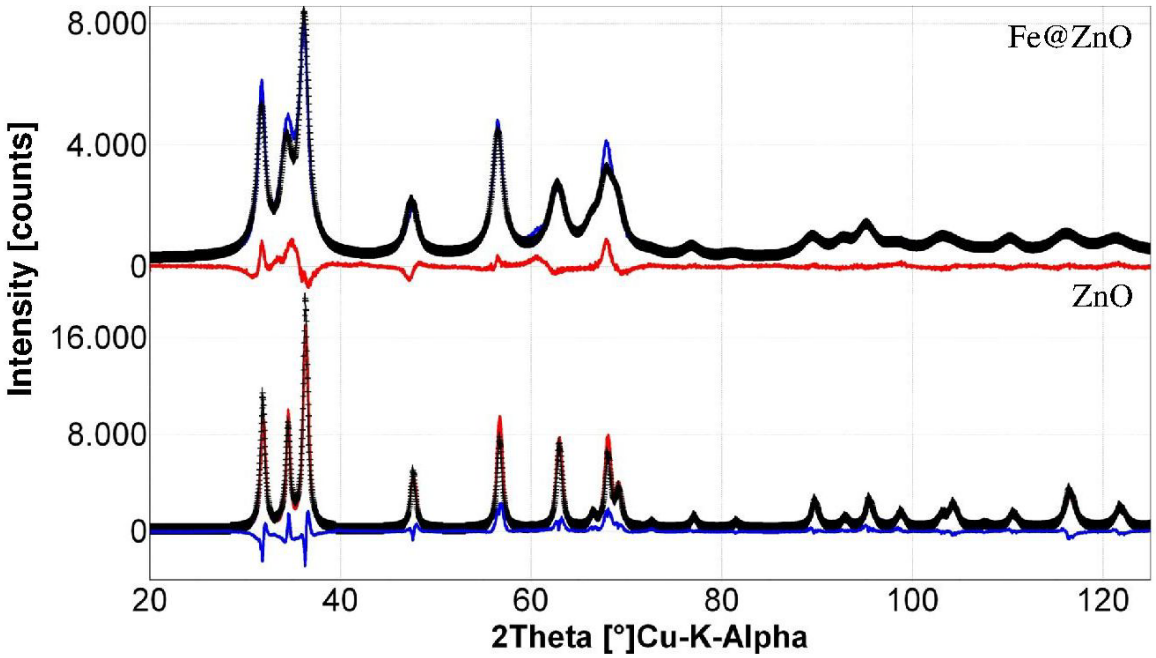


**Fig. 3.1** Rietveld refinement of pure and Fe@ZnO for the extraction of cell parameters and crystallite sizes. The cell parameters of ZnO: wurtzite structure, a=b= 3.2477, c = 5.2043, V = 47.54Å$^3$; Fe@ZnO: wurtzite structure, a=b= 3.2546, c = 5.2174, V = 47.86Å$^3$.

Representative images of pure and Fe @ZnO NPs are illustrated in Figure 2 (a)-(d). The morphology of as-synthesised Fe@ZnO have slightly spherical shape unlike pure ZnO, which has slightly elongated structure as shown in Fig. 2(a) and (d). The sizes of the particles were also evaluated using TEM images and the average particles sizes ($d_{TEM}$) were in the range of ~19 nm for ZnO and 12-13 nm for Fe@ZnO. The particle sizes ($d_{TEM}$) reasonably agree with the sizes obtained through BET ($d_{BET}$) and XRD ($d_{XRD}$) measurements (Table 1).

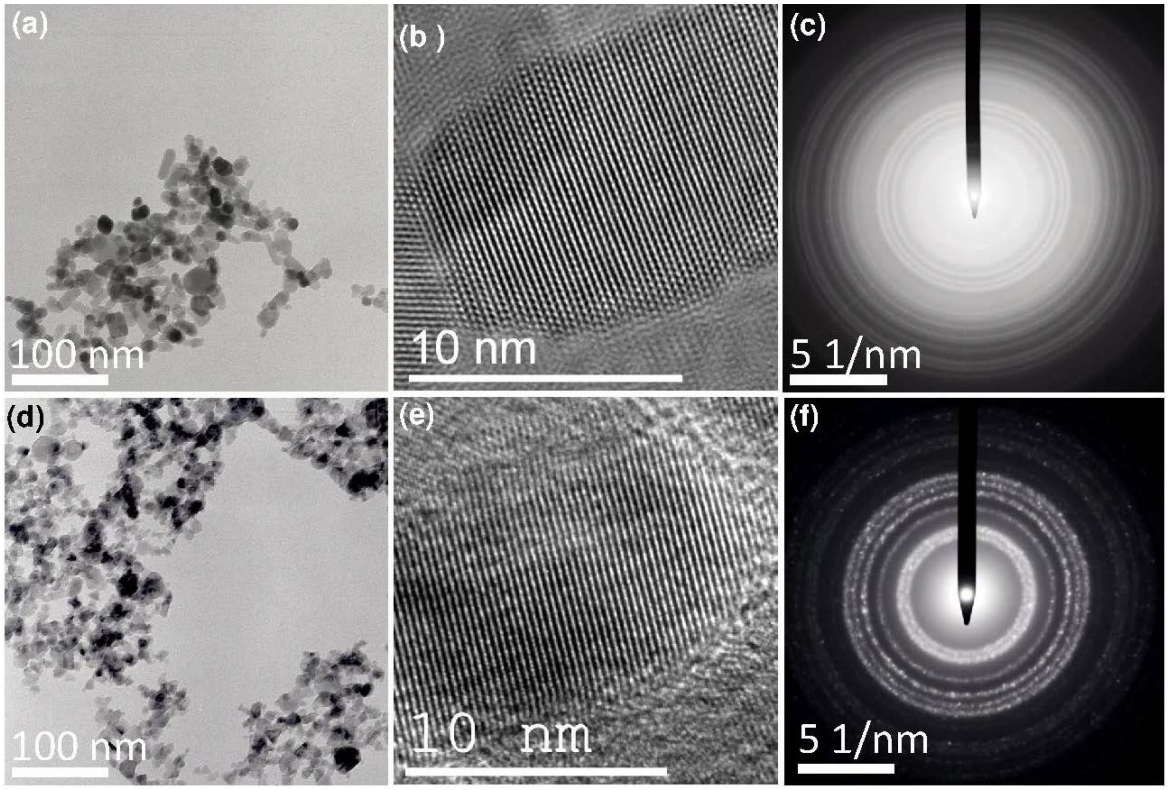


**Fig. 3.2** (a) and (d): Low resolution images showing particle morphology (b) and (e) single particle HRTEM images (c) and (f) selected area diffraction patterns of pure and 10%Fe doped ZnO NPs respectively. The results show that the doping is homogeneous and single crystalline with no detectable changes in the lattice spacings.

With long time sonication, ZnO NPs formed stable aggregates of ~200-300 nm size in DI water, except for Fe@ZnO, which aggregated continuously over 30-min to ~1100 nm. Electrophoretic mobility of the ZnO and Fe@ZnO exhibited positive mobilities ($1.45(\pm 0.29) \times 10^{-8}$ m/V· s for ZnO and $0.97(\pm 0.11) \times 10^{-8}$ m/V·s for Fe@ZnO) when dispersed in DI water [3].

### 3.3.3. Effect of 10% Fe@ZnO on seed germination and plant growth

In the present study, none of the treatments of Fe@ZnO NPs affected the rate of germination compared to control and no observable toxicological response was detected. However, previously reported studies have shown reduced seed germination with bare ZnO NP treatments in corn [37]. The difference could be attributed to lower release of $Zn^{2+}$ ions from 10% Fe@ZnO NPs (less than 1.5 mM at pH 7 after 16 h in water) [24].

The changes in root and stem lengths of pea plants treated with Fe@ZnO NPs are shown in Fig. 3. Although there was a numeral reduction in root length (33% reduction at 500 mg/kg treatment compared to control), the differences were not high enough to reach statistical significance. Recently Mukherjee et al. reported increase in root and stem lengths of green pea treated with bulk and nano-ZnO at all concentrations (125, 250, and 500 mg/kg) [26]. The difference in toxicological behavior of different ZnO NPs may be due to lesser dissolution of 10% Fe@ZnO NPs compare to that of bare ZnO NPs [24].

Reduction in relative chlorophyll content was observed in green pea leaves (Fig. 4). The difference reached statistical significance with 27% reduction in 500 mg NP/kg treatment. These results are in accordance with the uptake data, where 500 mg/kg NP treatments (Fig. 5) showed 72% increase in zinc bioaccumulation in leaves compared to control. Excess amount of $Zn^{2+}$ can destroy the chlorophyll units by substituting its central metal atom ($Mg^{2+}$), leading towards the

breakdown of the photosynthetic core followed by reduction in chlorophyll content and phytotoxicity [15].

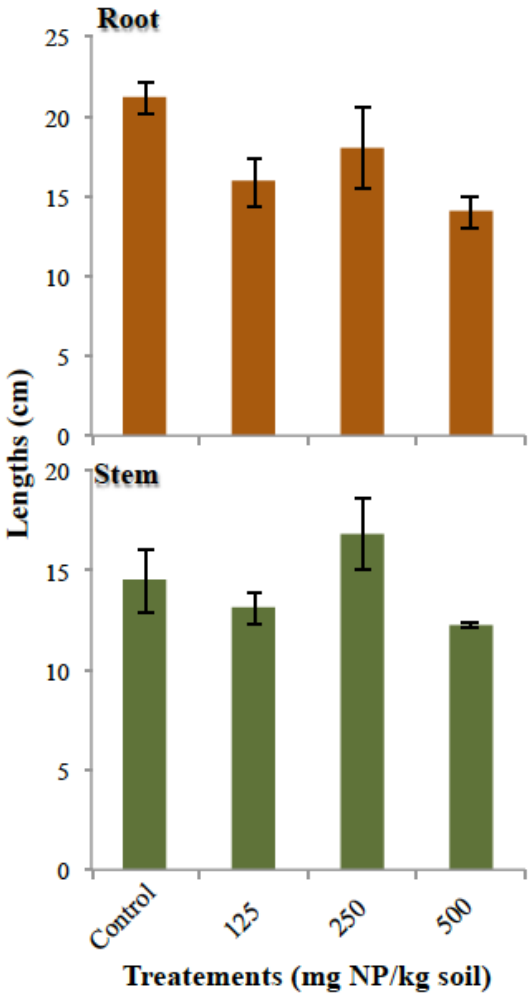


**Fig. 3.3** Root and stem lengths (cm) of green pea plants grown for 25 days in soil, treated with 0(control)-500 mg/kg Fe@ZnO NPs. Bars represent mean of four replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$.

Previous study reported that ZnO NP and bulk-ZnO treatments showed significant reduction in chlorophyll content (at 125, 250, and 500 mg $kg^{-1}$) [26]. However, in case of 10% Fe@ZnO NP treatments, reduction in chlorophyll content was observed only at the highest concentration (500 mg/kg). Lower dissolution of 10% Fe@ZnO NPs compared to that of undoped ZnO NPs and the bulk form is probably one of the key factors for lower toxicity of the doped NP. From the above discussion, it may be inferred that Fe doped ZnO NPs showed less phytotoxicity compared to that of ZnO NPs based on relative chlorophyll content [26].

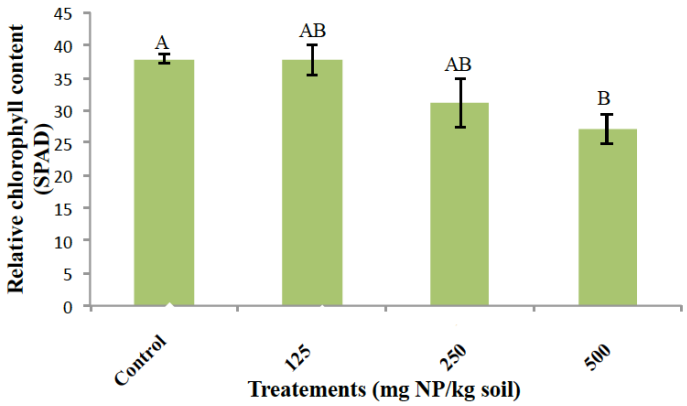


**Fig. 3.4** Chlorophyll contents (SPAD) in the leaves of green pea plants grown for 25 days in soil, treated with 0(control)-500 mg/kg Fe@ZnO NPs. Bars represent mean of four replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$.

### 3.3.4. Accumulation of Zn and Fe in different plant tissues

Accumulation of Zn and Fe in green pea plant tissues treated with Fe@ZnO NPs is shown in Fig. 5. Root tissues showed ~2x higher Zn accumulation in 500 mg/kg exposure than that of 125 and 250 mg/kg. In stem, Zn uptake increased by 31, 36, and 48% at 125, 250, and

500 mg/kg treatments respectively, compared to control. However, in leaves, only 500 mg/kg treatment showed significantly higher Zn (72%) than that of control. Conversely, 9x and 4x higher Zn accumulation was reported in green pea roots treated with 500 and 250 mg/kg undoped/bare ZnO NPs [26]. Authors also found 4x and 3x higher Zn in stem and leaf of green pea treated with 500 mg/kg bare ZnO NPs [26]. We hypothesize that this could be due to low Fe@ZnO NPs uptake in the plants than bare ZnO NPs. The larger size and lesser dissolution of Fe@ZnO NPs compared to that of ZnO NPs [24] making it less bioavailable to green pea plants, leading towards lower Zn accumulation.

**Table 3.2** Bioaccumulation factors under different treatments

| | **125 mg/kg** | | | **250 mg/kg** | | | **500 mg/kg** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Root** | **Stem** | **Leaf** | **Root** | **Stem** | **Leaf** | **Root** | **Stem** | **Leaf** |
| **Bioaccumulation factors** | 1.8 | 0.44 | 0.28 | 0.74 | 0.24 | 0.15 | 0.72 | 0.15 | 0.09 |

The rate of translocation of zinc (i.e., zinc concentrations in stem to those in roots) for control, 125, and 500 mg/kg treatments were: 0.73, 0.25, and 0.20. The bioaccumulation factors (concentration of Zn in plant over the initial concentration of Fe@ZnO NPs in the growth media) [26] were found to decrease in all parts of the plants with increasing NP concentration (Table 2) [38]. This clearly indicates that most of the Zn taken up was retained in roots. One reason might be that Zn was taken up in its nano-form (Fe@ZnO), and precipitated in cell walls and intercellular spaces. This possibility can be further verified by looking into the size of the NPs

(mean diameter ~1470 nm in water), which makes them difficult to transport through the vascular system. Similar decrease in translocation was observed in corn roots when treated with ZnO NPs [28].

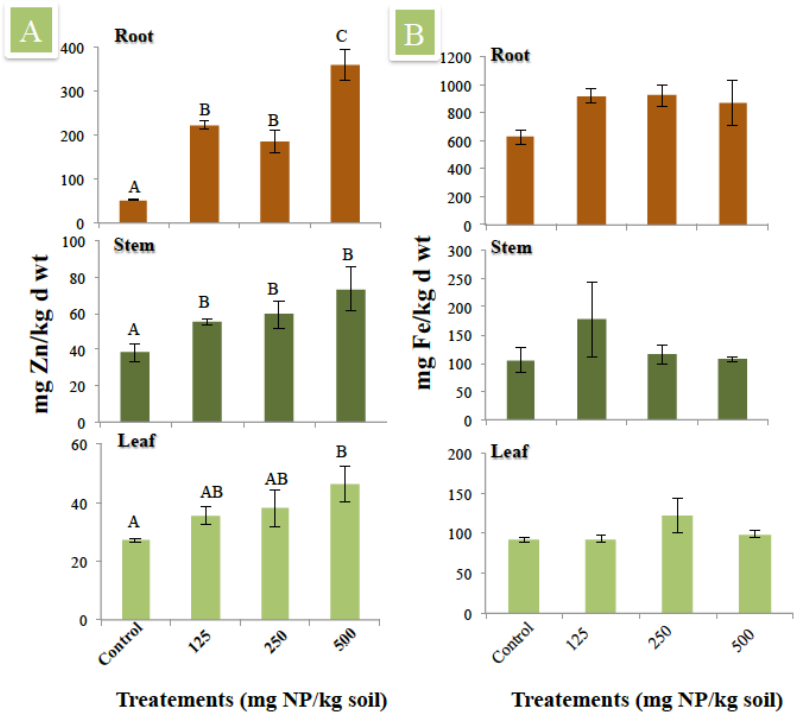


**Fig. 3.5** A) Zn and B) Fe concentrations in roots, stems, and leaves in green peas grown for 25 days in soil, treated with 0(control)-500 mg/kg Fe@ZnO NPs. Bars represent mean of four replicates ± standard error. Bars with the same/no letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

Fig. 3B shows that there is no statistically significant difference in Fe uptake in roots, stems, and leaves in all the treatments compared to control. This might be due to the fact that in Fe@ZnO NPs, the Fe loading is 10%. Furthermore, PXRD data shows that Fe is situated inside

the crystal lattice of ZnO, which might lowered its dissolution, causing less availability in the growth media.

### 3.3.5. $H_2O_2$ generation induced by Fe@ZnO NPs in roots, stems, and leaves

Natural cellular activities produce reactive oxygen species (ROS). However, there is a gentle equilibrium between their production and destruction/removal from the system [39-41]. In the present study, only roots at 500 mg/kg treatment showed ~50% decrease in $H_2O_2$ compared to all other treatments (Fig. 6). Lower $H_2O_2$ in roots signifies lesser ROS production leading towards less stress in green pea roots.

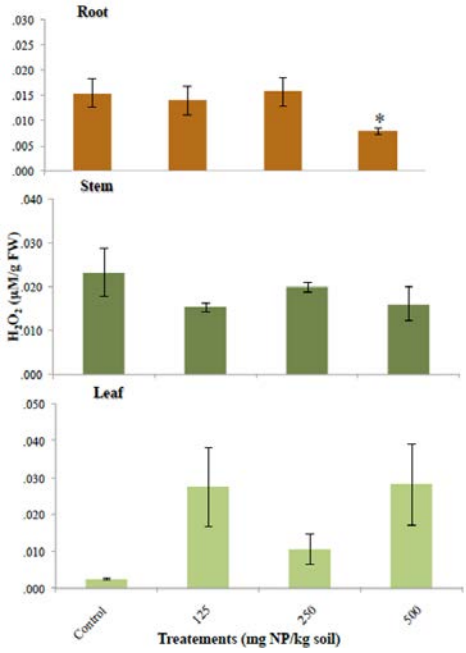


**Fig. 3.6** $H_2O_2$ concentration in roots, stems, and leaves of green pea plants grown for 25 days in soil, treated with 0(control)-500 mg/kg Fe@ZnO NPs. Bars represent mean of four replicates ± standard error. Bar with "asterics" shows statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

In addition, $H_2O_2$ generation in stem and leaf were unaffected by Fe@ZnO treatments. However, bare ZnO NPs showed overproduction (61%) of leaf $H_2O_2$ at 500 mg/kg treatment [26], confirming elevated stress level compared to that of Fe@ZnO NPs.

### 3.3.6. $H_2O_2$-scavenging enzyme activities

The anti-oxidative enzyme activities of APOX and CAT were measured after 25 days of treatment. As shown in Fig. 7A, activity of APOX was significantly reduced in all the treatments compared to control. However, the change in APOX activity in roots, stems, and leaves were insignificant among all the treatments (at $p \leq 0.05$). In roots, there were 73%, 49%, and 33% decrease in APOX activity with 125, 250, and 500 mg/kg treatments, respectively, compared to control. In case of stem, a similar trend was observed. The reduction was 84%, 86%, and 74% with 125, 250, and 500 mg/kg treatments, respectively, compared to control. Mukherjee et al. reported similar trend, where bare ZnO NP decreased APOX activity in root and leaf of green pea plants [26]. This nature of the curve can also be correlated from a previous study performed by Hernandez-Viezcas et al. in velvet mesquite (*Prosopis juliflora-velutina*), where the authors reported, a similar trend (decreased by 73%, 66%, and 89% with 125, 250, and 500 mg/kg) in APOX activity with increasing ZnO NP concentration [42].

In roots and leaves, CAT activity was significantly increased at 500 mg/kg treatment compared to all other treatments (Fig. 7B). However, in stem, Fe@ZnO NPs did not affect the CAT activity. Green pea leaves showed elevated level of CAT activity at 250 and 500 mg/kg treatments, compared to control. These results are in accordance with the study performed by Hernandez-Viezcas et al. with velvet mesquite (*P. juliflora-velutina*), where increase in CAT activity only at the highest concentration (4000 mg/kg) [42]. However, decreased CAT activity was reported in green pea leaves with increasing bare ZnO NP doses, signifying higher ROS

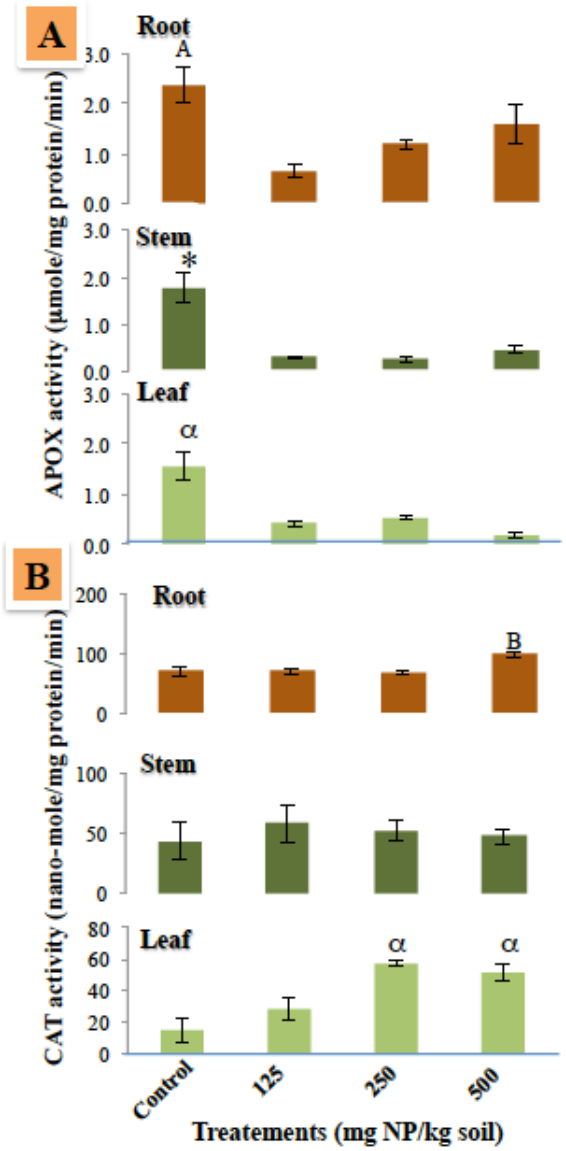


**Fig. 3.7** (A) APOX and (B) CAT concentrations in roots, stems, and leaves of green pea plants grown for 25 days in soil, treated with 0(control)-500 mg/kg Fe@ZnO NPs. Bars represent mean of four replicates ± standard error. Bars with the same letters/symbol show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

activity in leaves [26]. The differential behavior of bare and doped ZnO NPs could be attributed towards different size, surface charge, and dissolution properties of the NPs [24].

### 3.4 Conclusions

These findings confirm that Fe@ZnO NPs did not produce visible signs of toxicity including necrosis, stunting, chlorosis or wilting in all the treatments. However, Fe@ZnO NPs affected different physiological and biochemical parameters in terms of plant growth, chlorophyll content, ROS ($H_2O_2$) production, and antioxidative enzyme activity. Nevertheless, as per relative chlorophyll content and ROS production are concerned, we found that Fe@ZnO is less toxic than ZnO NPs in green peas grown under the same conditions. Key findings of this work support the fact that greater hydrodynamic diameter and/or lesser dissolution from Fe@ZnO NPs make it less available for green pea plants, and hence lesser phytotoxicity compare to that of bare ZnO NPs. Therefore, iron doping may be considered as an effective alternative approach to reduce the toxicity of ZnO NPs in higher terrestrial plants. Future studies have to be performed in wider range of NP concentrations to determine the mechanisms of toxicity and the effects on seed quality.

# Chapter 4

## A life cycle comparative phyto-toxicological study of bare ZnO, alumina doped ZnO ($Al_2O_3$@ZnO), and KH550 coated ZnO (KH550@ZnO) nanoparticles in green pea plant (*Pisum sativum* L.) and its seed quality


### Abstract

In this study we performed phyto toxicological studies of nanoparticles (NPs) on green pea, one of the highest consumed legumes in the world. Green pea plants were grown in soil treated with three different NPs, e.g., bare ZnO NPs (10 nm), 2 wt% alumina doped ($Al_2O_3$@ZnO NPs, 15 nm), and 1 wt% aminopropyltriethoxysilane (KH550 or silane coupling agent) coated (KH550@ZnO NP, 20 nm) NPs along with bulk (ZnO) and ionic Zn (zinc chloride) at 250 and 1000 mg/kg for 65 days. Upon harvest, fresh and dry weights, zinc, aluminum, and silicon uptake was determined. Chylorophyll *a*/*b* and carotenoid concentrations were also measured. Results showed that the fresh and dry weights were not affected by all the treatments (except with coated 100 mg/kg treatment). The zinc uptake in roots and stems increased in a concentration dependent manner (except with bulk & ionic treatments in roots; and only ionic in stem). However, the doped treatment showed the highest uptake (37x in root and 20x in stem) among all the treatments, compared to control. In leaves, doped treatments also showed the highest accumulation (11x) at 1000 mg/kg treatment. Aluminum and silicon uptake remained mostly unaffected. In leaves, doped treatments also affected the Chl-*a* and carotenoid concentrations, keeping the Chl-*b* concentrations unaffected. The results confirmed that in spite of larger size (15 nm) of the doped ZnO NPs, these NPs showed more effects than that of the bare ZnO NPs (10 nm).



**Key words:** bare, doped, coated ZnO Nanoparticles, bulk ZnO, Zn uptake, phyto-toxicity

## 4.1 Introduction

According to the U.S. National Nanotechnology Initiative (NNI), "Nanotechnology is science, engineering, and technology conducted at the nanoscale, which is about 1 to 100 nanometers."(*1*). Due to their high surface to volume ratio and higher number of atoms at the grain boundaries, engineered nanoparticles (ENPs) have been widely used in the fields of, but not limited to, medicine, agriculture (nano-fertilizers and nano-pesticides), manufacturing, electronics, and energy production is prominent (*2-5*). Due to their worldwide use is one of the most prominent routes of environmental exposure to ENPs. Unique properties like high reactivity and bio-compatibility of NPs are two of the major causes of their potential toxicity to living organisms. Recent literature has shown that various animals, plants, and microorganisms could be affected by ENP exposure (*6-12*). Reports describe the toxicity of ENPs on crop plants, such as: cucumber (*Cucumis sativus*), radish (*Raphanus sativus*), lettuce (*Lactuca sativa*), mungbean (*Phaseolus radiatus*), wheat (*Triticum aestivum*), rape (*Brassica napus*), corn (*Zea mays*), and alfalfa (*Medicago sativa*), among others (*6-17*). However, mechanistic understanding of the impact of ENPs on edible/crop plants is mostly unknown.

Among all the widely used NPs (e.g., silver, carbon, titanium dioxide, silica, zinc, and gold) zinc (and its oxide) NPs secure fifth position in terms of its consumption in the global market (*18*) "The Nanotechnology Consumers Products Inventory" identified ZnO NPs as one of the widely used NMs (*18*). Zn oxide nanoparticles (ZnO NPs) are one of the most used NPs in different commercial and industrial processes throughout the world and considered as potentially toxic to the environment (*19*). ZnO NPs are used in personal care products (mainly in sunscreens), anti-microbial agents, paints, and photovoltaics, among others (*20-22*). However, the end user residues route to the environment and shows adverse effects on living organisms

including plants. Studies have shown that these NPs have differential toxic effects in plants. For instance, root elongation in ryegrass (*Lolium perenne*), radish (*Raphanus sativus*), and rape (*Brassica napus*) had been reported by Lin and Xing (2007) with the treatment of ZnO NPs (*21*). These NPs can impose phytotoxicity by disrupting the water and nutrient pathways in plants (*21*, *23*). Reduction of biomass in wheat (*Triticum aestivum*) with an elevated ROS level has been reported by Dimpka et al. (*29*). There are also reports on changes in germination of desert plants (*29*). Lopez-Moreno et al. reported the genotoxic nature of ZnO NPs in soybean plant (*Glycine max*) (*25*). Toxicity of ZnO NPs seems to be due to its greater dissolution in the growth media (release of $Zn^{2+}$ ions) and/or by the induction of oxidative stress by the NPs *per se* (*26-30*). Zhao et al. reported reduction in chlorophyll production on corn plants (*Zea mays*, golden variety) grown in soil treated with ZnO NPs at 800 mg/kg (*31*). Released $Zn^{2+}$ ions from the dissolution of ZnO NPs can displace the central metal atom $Mg^{2+}$of chlorophyll, and hence, destroy the photosynthetic core, which, in turn, causes phytotoxicity (*33-36*). Previous studies have shown that ZnO NP treatments can enhance reactive oxygen species (ROS) production, leading towards oxidative stress (*37*,*38*). To battle with these ROS molecules, plants produce different antioxidative enzymes, which interact with the ROS molecules and convert them into less harmful byproducts (*39*). There are various different antioxidative enzymes present in plant tissues, e.g., ascorbate peroxidase (APOX), catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), guaiacol peroxidase (GPOX), and dehydroascorbate reductase (DHAR), among others (*40*).

In recent years, hybrid NPs, e.g., doped and coated NMs, are getting increasing attention due to their potential applications in microelectronics, semiconductors, optical device fabrication, optics, and electricity (*41-44*). Silane coupling agent (KH550) coated ZnO NPs and

alumina doped ($Al_2O_3$) ZnO NPs are two of the most important in their class. Therefore, it is of utmost importance to measure the phyto-toxicological effects of bare, coated, and doped ZnO NPs.

Green pea is one of the most important legumes in terms of worldwide production and consumption. They are rich in protein, certain minerals, and vitamins with low calorific value (*44*). Raw green peas are excellent source of vitamin K, C, B1, B9, A, B6, B3, and B2. It is also rich in Mn, P, Mg, Cu, Fe, An, and K (*44*). Among four major legumes (i.e., lentil, green peas, chickpea, and cow pea) green pea is the second best protein source (24.9 g/100 g raw green pea) (*44*). It has been reported that a cup of raw green peas (= 137.75g) provides 30.3% fiber, 14.7% of protein, and only 6% calories (daily nutritional value) (*44*).

In spite of having high nutritional value and global consumption, to the best of author's knowledge, there is no report of comparative toxicological studies on the interaction of green pea plants and bare, coated, and doped ZnO NPs. Therefore, there is an urgent need to study the phyto-toxicological responses of different ZnO NPs on green pea plants, especially on grain quality.

This work is aimed at shining light in this regard. Plants were exposed to different concentrations of NPs and bulk (125, 250, and 500 mg/kg soil), and control experiment where no NPs were applied. The NPs were characterized prior to application by measuring the dissolution of all the NPs (including bulk) in soil solution and the zeta potentials, pH, and sizes in MPW were recorded. Accumulation/uptake of zinc, aluminum, and silicon in different parts of the plants were measured through ICP-OES/ICP-MS. Leaf chlorophyll *a/b* and total carotenoids were measured spectro-photometrically. The green peas were analyzed for mineral contents,

carbohydrate, and protein profiles. This work might have potential to illustrate the effects of crystal and/or surface modification of ZnO NPs on higher terrestrial plants, like green peas.

## 4.2 Materials and Methods

### 4.2.1 Soil sampling

The original soil was collected from Horizon, TX (31°51′59.06″N; top 20 cm) and prepared as earlier. The soil type was sandy loam soil based on percentages of clay, silt, and sand of 3.73%, 12.15%, and 84.1%, respectively. The experiment was performed in a 1:1 mixture of the native soil with high organic matter soil [Miracle Gro® Potting Mix (Tierra para macetas; Marysville, OH)] to provde the enough supply of nutrient. The potting soil was purchased from a nursery to maintain appropriate pore size and increase soil fertility. The soil pH, CEC, and Zn concentration was measured after mixing.

### 4.2.2 Pot preparation (Mixing NPs with soil)

NPs and bulk ZnO were added solid at 0 (control), 250, and 1000 mg NPs/kg of soil in black plastic container (Ns-400; Diameter: 20cm; Tall: 12.5 cm; Volume: 3.925 L; Nursery Supplies, Inc.) with 1:1 ratio of local regular soil and Miracle Gro® Potting Mix (Tierra para macetas; Marysville, OH). Soil was vigorously mixed with spatulas to maximize the homogeneity of the particles. 200 ml nutrient solution from injector was added [0.72 $g \cdot L^{-1}$ 15 N- 2.2 P- 12.5 K (Peters 15-5-15) to tap water; (Control, EC = 1.80 dS/m; pH= 6.62)] and kept 24h for stabilization in the green house. The daily light integral (photosynthetically active radiation) was 15.3 ± 3.1 $mol \cdot m^{-2} \cdot d^{-1}$. The temperatures in the greenhouse were maintained at 26.9 ± 8.6 °C (mean ± standard deviation) during the day and 13.7 ± 4.3 °C at night. The relative humidity was 41.6 ± 19.1%.

### 4.2.3 Seed germination and exposure

Early rise varieties of green peas (life cycle of 65 days) were sown in soil. Seeds were washed in 4% bleach solution and rinsed three times with tap water. Seeds were soaked overnight in regular tap water. Next day, those seeds were sown in the test pots and left to germinate and grow for 65 days.

### 4.2.4 Dissolution of different NPs in soil solution

The ZnO NPs (10 nm commercial spheroid, Meliorum Technologies, New York) were obtained from the University of California Center for Environmental Implications of Nanotechnology (UCCEIN). 2 wt% $Al_2O_3$@ZnO (15 nm), and 1 wt% KH550 coated ZnO NPs (20 nm) from US Research Nanomaterials, Inc. (http://www.us-nano.com). All three different NPs and bulk ZnO were dispersed in soil solutions (containing 5 g 1:1 soil and 20 ml water) to achieve 1000 mg/kg concentration with respect to the weight of the soil. Each measurement was done with three replicates. Three set ups (for 15, 30, and 45 days measurements) were made and left them undisturbed (closed cap). Each set up was used for the dissolution study at a particular time interval. Periodically, 50 ml tubes were centrifuged at 5000 rpm (Eppendorf AG bench centrifuge 5417R, Hamburg, Germany), 2 ml supernatant was taken out and centrifuged at 14000 rpm, and finally, 1 ml supernatant was taken out and centrifuged at 14000 rpm. Multiple time serial centrifugations were done to remove the particles from the solution to get the actual concentration of the dissolved ions. Final supernatant was diluted to 15 ml with 4% $NHO_3$ and elemental concentrations were measured using ICP-OES.

### 4.2.5 Zeta potential, size, and pH of the NP suspensions

Particles were dispersed in 10 ml MPW to achieve 250 and 1000 mg/kg concentrations. The samples were kept undisturbed for 1 h and measured the zeta potential and size using Nano-

ZS 90, Malvern Zetasizer. pH of the supernatants were measured using a pH meter. Each experiment was performed in three replicates.

### 4.2.6 Zn Uptake in plant and seed

After 65 days, upon harvest, plants were washed with 0.01 M $HNO_3$ and rinsed with DI water. Thereafter, roots, stems, leaves, and seeds were separated and oven dried at 70°C for two days (Fisher Scientific Isotemp; 4914 Baum Blvd, Pittsburg, PA; USA), weighed, and digested with plasma pure$HNO_3$ and $H_2O_2$ (1:4) as described by Packer et al. (*46*), with little modifications. The digested samples were analyzed using a Perkin Elmer 4300 DV inductively coupled plasma optical emission spectrometer (ICP-OES) or ICP-MS (ELAN DRC II; Perkin-Elmer) as required.

### 4.2.7 Chlorophyll and carotenoid estimation

Approximately 0.5 gram fresh, razor blade chopped plant leaves were placed into 15mL tube. Five ml pure acetone was added and shaken overnight in a horizontal shaker (Revco Scientific Inc. Model# DS1473AVA, 115 volts, 60 Hz, 7 amps.). The supernatants were collected and absorbance measured at 470, 645, 662 nm using a Perkin Elmer Lambda 14 UV-vis spectrometer (single-beam mode, Perkin-Elmer, Uberlinger, Germany. Concentrations of Chl-*a*, *b*, and total carotenoids were measured according to Kumar et al. (*49*).

### 4.2.8 Determination of total soluble, reducing sugars, and starch

The total soluble sugars extraction was performed following the method of Verma and Dubey (48) with little modifications. A sample of 100 mg of dried (inside the fume hood) green pea seeds was ground in 2 ml of 80% ethanol and then boiled (80°C) in a water bath for 30 min. After cooling down to room temperature, the extracts were centrifuged at 14000*g* (Thermo Scientific, Soruall T1, U.S.A.) for 30 min and this process was repeated two times. All the

supernatants were combined. Using this extracts, soluble sugar content was determined following the method of Dubois et al. (*47*). Reducing sugar content was determined by the procedure of Somogyi (*58*). The amount of non-reducing sugar was determined by subtracting the value of reducing sugar from total sugar.

The starch was extracted following the method of Verma and Dubey (*46*). The residue from total sugar extraction was used to determine the starch content. Precipitate was dried at 70°C for 24 h, added 2 mL of MPW, and the mixture was boiled in a water bath for 15 min. After cooling down to room temperature, 1 mL of concentrated sulfuric acid was added. The suspension was stirred for 15 min, and the final volume was adjusted to 5 mL using MPW. The supernatant was centrifuged at 3000*g* for 20 min, and the extraction was repeated once using 50% sulfuric acid. The supernatants were combined and diluted up to 10 ml. The starch content was quantified following the method of Dubois et al. (*47*) and expressed in mg/100 g dry weight.

### 4.2.9 Protein fractionation

Protein fractionation was performed according to Chen and Bushak (*50*). Dried green pea seeds (100 mg) were extracted sequentially with 2 mL each of water, 0.5 mol/L NaCl, 70% ethanol, and 0.05 M acetic acid for 2 h. The extracted protein in each step was labeled as albumin (water soluble), globulin (salt-soluble), prolamin (alcohol-soluble), and glutelin (acid-soluble), respectively. Each fraction was centrifuged at 4000g. Supernatants were collected and analyzed using Bradford method (*51*) explained earlier.

### 4.2.10 Statistical analysis

All the treatments were replicated four times. Data (means ± SE) were reported as averages of four replicates. A one-way ANOVA test was performed, and Tukey-HSD multiple

comparisons conducted test performed using the statistical package SPSS version 12.0 (SPSS, Chicago, IL) at $p \leq 0.05$.

### 4.3 Results and Discussion

#### 4.3.1 Particle dissolution

After 15 days, bare, doped, and coated ZnO NPs showed significant higher dissolution compared to that of bulk ZnO. At 15th day, the amount of dissolved zinc was up to 1.1 ppm in case of doped NPs. However, slightly less dissolution in nano (0.86 ppm) and coated (0.9 ppm) was observed in MPW but this difference was not enough to reach statistical significance. On the other hand, bulk ZnO particles showed 0.37 ppm zinc dissolution which was considerably less than that of other NPs. This can be attributed to the very large size and small surfacea area of bulk particles, which enormously decreases the surface area and hence reduce the dissolution of bulk ZnO compared to NPs (Fig. 4.1). The amount of dissolved zinc remained unaltered after 30 and 45 days. This might due to the fact that dissolved zinc ions produce zinc hydroxide, which can precipitate out from the system, leaving behind fewer amounts of zinc ions in the solution. Silicon and aluminum concentrations remained unaltered during the entire period of time. This might be attributed to very low availability of aluminum and silicon in doped (only 2 wt %) and coated (only 1 wt % KH550) respectively.

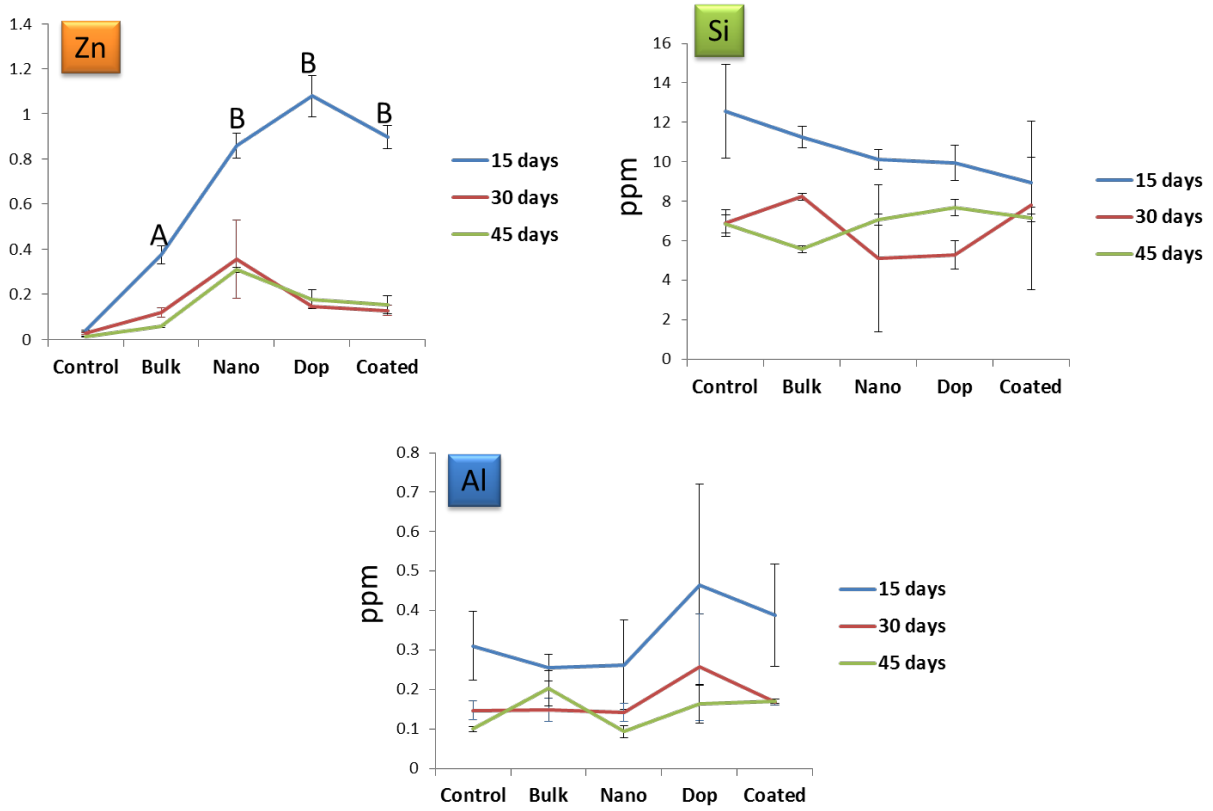


**Fig. 4.1** Zinc, silicon, and aluminum dissolution of all the particles after 15, 30, and 45 days at 1000 mg/kg concentration. Data points with same letters represent no statistical significance at $p \leq 0.05$

### 4.3.2 Size, zeta potential, and pH

In MPW solution, at both 250 and 1000 ppm, doped NPs showed less hydrodynamic diameter than that of others (Table 4.1). The size distribution of dispersed NPs were in order of : doped NPs < bare-NPs < coated NPs << bulk ZnO. At 250 and 1000 ppm dispersions, bulk (1627±198.9 nm and 9324±236.8 nm) showed higher diameter than that of doped (362.2±20.7 nm and 244.1±25.6 nm), bare-ZnO (397.5±25.3 nm and 290.9±20.2 nm), and coated (526.6±14.2 nm and 608.5±11.9 nm). As the concentration increased, the size of the particles became smaller (except coated and bulk). This may due to higher rate of precipitation and co-precipitation at 1000 ppm compared to that of 250 ppm, leaving behind smaller particles.

**Table 4.1** Zeta potential, pH, hydrodynamic diameter of different particles. Each measurement has three replicates. Data are mean±SE ($p \leq 0.05$).

**Zeta potential (mV)**

| Concentration (mg/kg) | Bare-ZnO | Coated | Dop | Bulk |
|---|---|---|---|---|
| 250 | 23.6±3.89 | -15.3±7.57 | 27.7±4.89 | 28.7±4.55 |
| 1000 | 20±4.57 | -26.4±7.09 | 23.9±3.74 | 27.7±5.36 |

**pH**

| Concentration (mg/kg) | Bare-ZnO | Coated | Dop | Bulk |
|---|---|---|---|---|
| 250 | 8.0±0.01 | 7.9±0.03 | 7.7±0.05 | 7.9±0.02 |
| 1000 | 8.5±0.01 | 8.0±0.02 | 7.7±0.09 | 7.9±0.08 |

**Size (nm)**

| Concentration (mg/kg) | Bare-ZnO | Coated | Dop | Bulk |
|---|---|---|---|---|
| 250 | 397.5±25.3 | 526.6±14.2 | 362.2±20.7 | 1627±198.9 |
| 1000 | 290.9±20.2 | 608.5±11.9 | 244.1±25.6 | 9324±236.8 |

All the particles showed positive zeta potential (except the coated one). The order of magnitude was: coated (-ve) < bare-ZnO < doped < bulk. The higher zeta potential for doped NPs compared to bare-ZnO NPs can be attributed to the fact that $Al^{3+}$ replaced $Zn^{2+}$ in the ZnO lattice, which increases the surface potential. The negative zeta potential of the coated one can be explained by looking into the nature of the surface coating. Aminopropyltriethoxy-silane (KH550) has one amine and three ethoxy groups (both the groups have electronegative centers). Probably, this is why, attachment of KH550 onto the surface of ZnO NPs creates a negative surface charge (by the oxygen atoms), leading towards negative zeta potential. The pH of the

suspensions (7.7 to 8.5) did not change considerably with different concentrations or the nature of the particle.

**Table 4.2** Elemental analysis of native and 1:1 native soil: potting mix. Samples were analyzed in three replicates. Data are mean±SE ($p \leq 0.05$).

| Parameters | Native soil | 1:1 soil |
|---|---|---|
| pH | 7.7±0.18 | 7.2±0.08 |
| Zn | 39.4±2.4 | 87.2±4.8 |
| Al | 7542±43 | 6124±178 |
| K | 8713±249 | 12460±89 |
| Ca | 10784±100.9 | 12716±247 |
| Fe | 7515±48 | 4578±167 |
| Mg | 2711±145 | 4591±57 |
| S | 719±47 | 1451±41 |
| Mn | 57±9.14 | 89.14±4.9 |
| P | 39±1.3 | 108±4.8 |
| Cu | 14.5±4.1 | 19.0±0.9 |
| Mo | 1.1±.09 | 2.9±0.2 |

### 4.3.3 Fresh/dry weights and zinc/aluminum/silicon bioaccumulation in root, stem, and leaf

Zinc and aluminum concentrations in native soil were 39.4±2.4 and 7542±43 mg/kg and 87.2±4.8 and 6124±178 in 1:1 soil. The soil pH was slightly lower in 1:1 soil than that of native soil may be because of the presence of organic acids in the potting soil (Table 4.2). No change in

plants fresh and dry weights with treatments except 1000 mg $kg^{-1}$ ZnO@KH550 treatment, which showed about one fold (95%) increase in plant fresh weight compare to control (Figure 4.2).

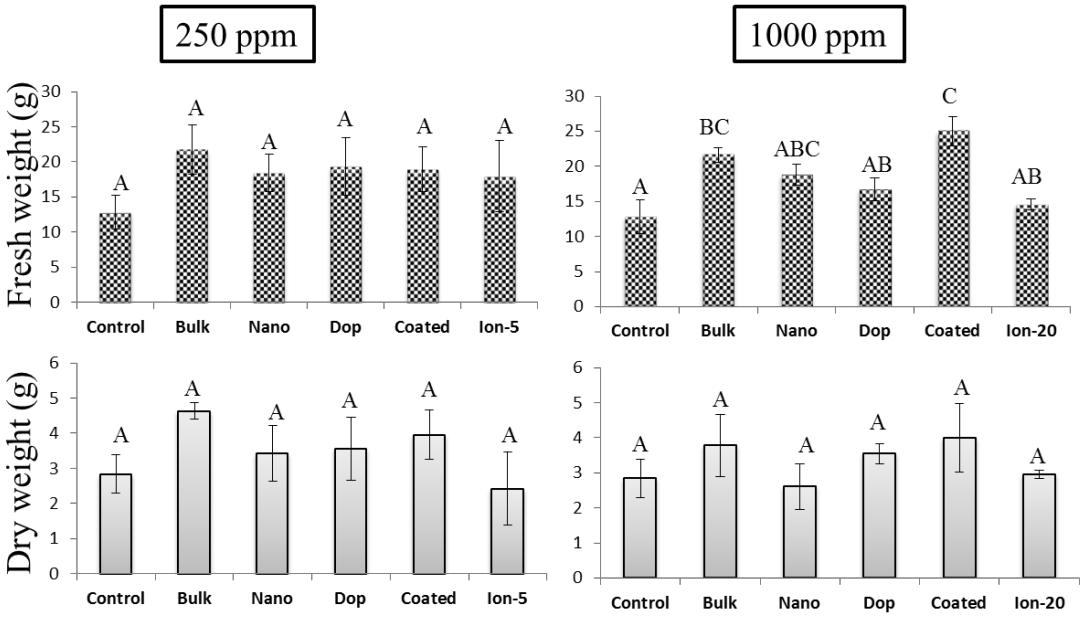


**Fig. 4.2** Fresh and dry weights of the total plants. Bars with same letters represent no statistical significance at $p \leq 0.05$.

Plant roots showed significant increase in Zn accumulation of 5.7x, 5.7x, and 8x treated with 250 mg $kg^{-1}$ bulk ZnO, bare ZnO NP, and $Al_2O_3$@ZnO NP, respectively, compared to control. Similarly, 1000 mg $kg^{-1}$, bare ZnO NP and $Al_2O_3$@ZnO NP treatments showed significant increase up to 16 and 36 times compared to control (Figure 4.3). A concentration dependent increase in Zn uptake was observed in 250 and 1000 mg/kg treatments with 3x, 2.8x, and 4.6x increase in nano, coated, and doped respectively (Fig. 4.3).

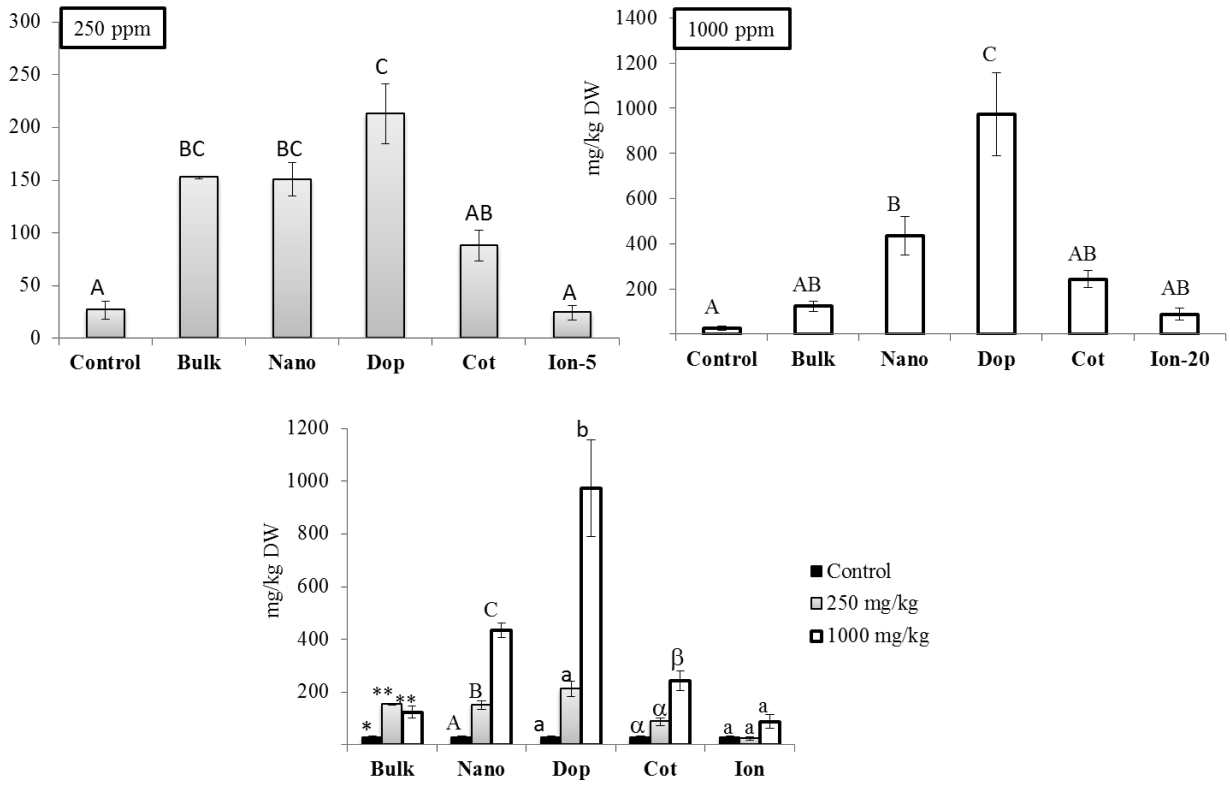


**Fig. 4.3** Zinc bioaccumulation in root tissues. Bars with same letters/symbols represent no statistical significance at $p \leq 0.05$.

Green pea stems showed higher level of Zn accumulation except with the ionic zinc treatment. Increase in Zn accumulation was in the following order: at 250 mg $kg^{-1}$: bulk (5x), bare (7x), doped (4.7x) and coted (7x); at 1000 mg $kg^{-1}$: bulk (9x), bare (11x), doped (20x) and coted (9x)] compared to control (Fig. 4.4). However, in between treatments, bulk (1.8x), bare (1.5x), and doped (4.3x) showed increase in zinc uptake in a concentration dependent significant. In leaves, all the treatments (bulk and coated) showed significant increase in zinc uptake (4.6x to

5.3x), except at 250 mg $kg^{-1}$ and 500 mg $kg^{-1}$ treatments. In leaves, 1000 mg $kg^{-1}$ treatments (bulk, bare, and doped) also showed significant increase in zinc uptake (5.5x to 11x) except for coated and ionic treatments (Figure 4.5). In leaves, the highest uptake of doped NPs might be attributed to it smaller size and higher dissolution of compared to that of others particles.

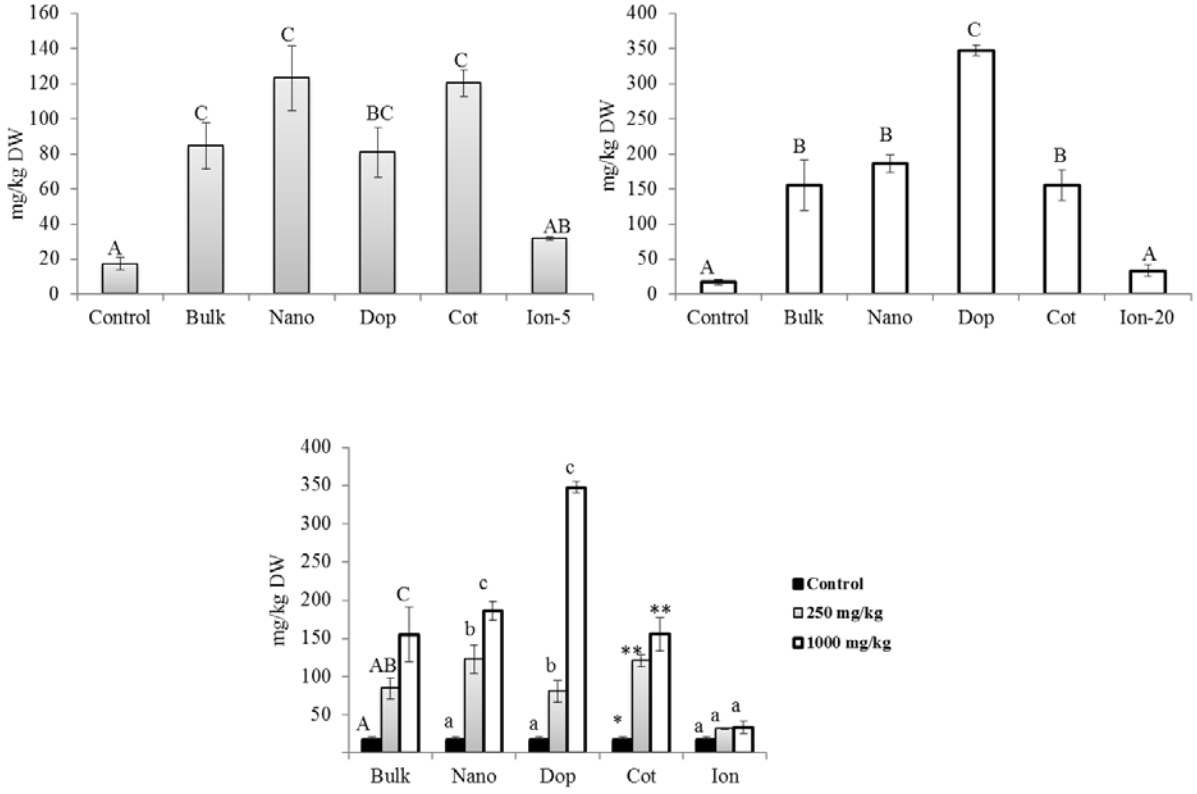


**Fig. 4.4** Zinc bioaccumulation in stem tissues. Bars with same letters/symbols represent no statistical significance at $p \leq 0.05$.

Aluminum and silicon uptake did not change with few exceptions at 1000 mg $kg^{-1}$. At 1000 mg/kg treatments, doped (2.7x) and coated (3.3x) NPs showed significant decrease in Al uptake in stems (Fig. 4.6). However, silicon uptake was decreased significantly at 250 mg/kg bare (2.6x)

and doped (2x) treatments (Fig. 4.7). In roots, bare showed a decrease (2.4x) but doped treatment showed an increase (22%) in Si uptake at 1000 mg/kg treatments compared to control.

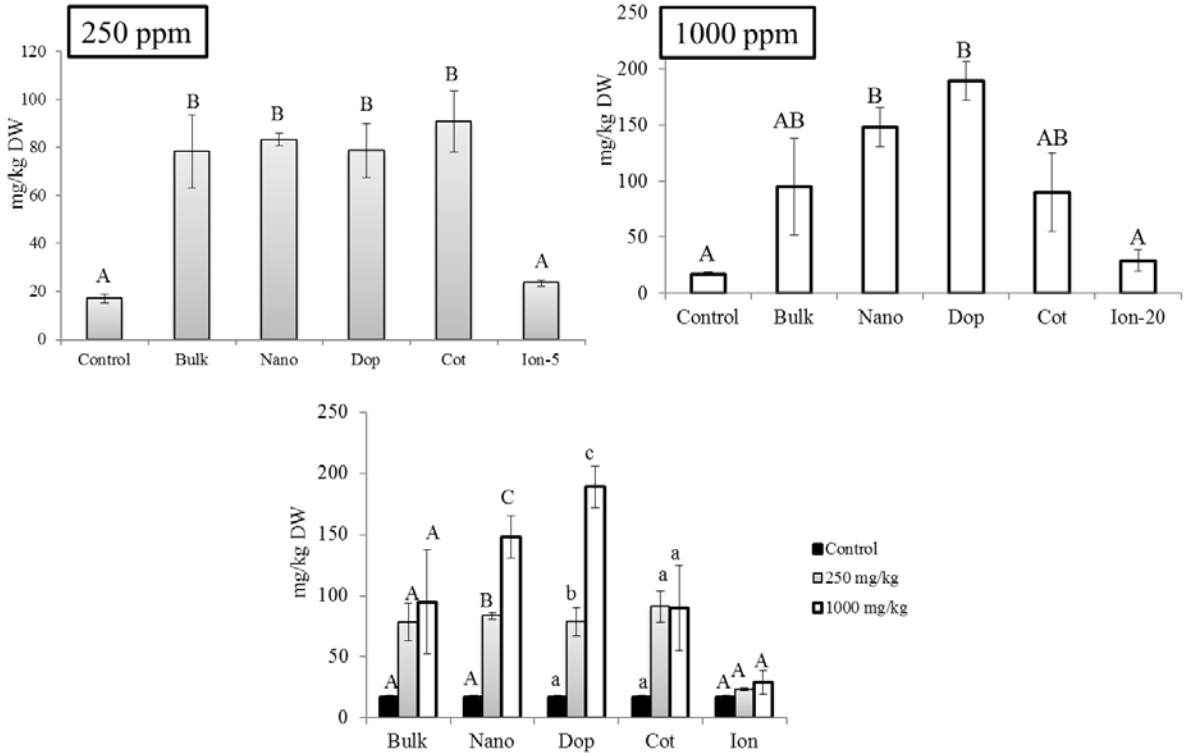


**Fig. 4.5** Zinc bioaccumulation in leaf tissues. Bars with same letters represent no statistical significance at $p \leq 0.05$.

Al and Si are not considered essential nutrients in plants; however, we wanted to confirm the possible uptake of these two elements with Zn in nano form or ionic/dissolve Zn form. Our results indicate that increase or decrease of aluminum and silicon did not resonate with zinc uptake. Therefore, probably, mineral uptake through dissolution is overshadowing the uptake and translocation of NPs in different plants tissues.

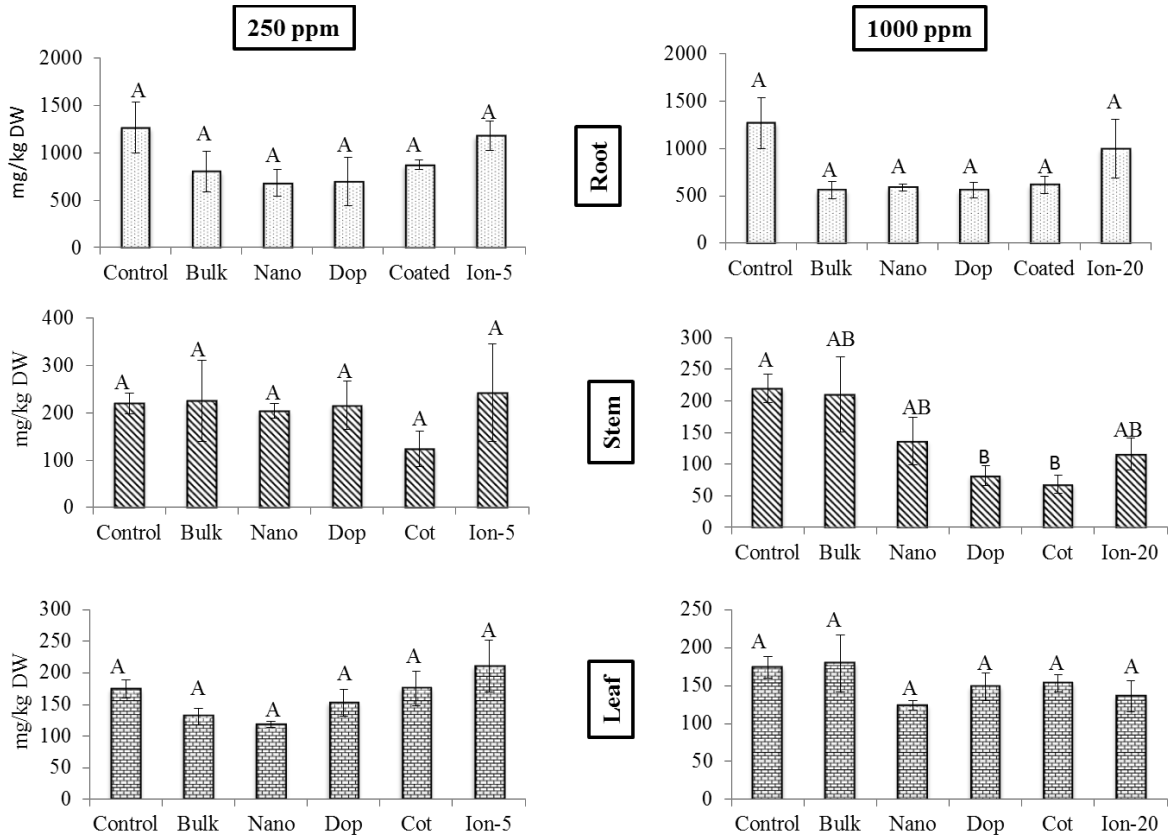


**Fig. 4.6** Aluminum bioaccumulation in root, stem, and leaf tissues. Bars with same letters represent no statistical significance at $p \leq 0.05$.

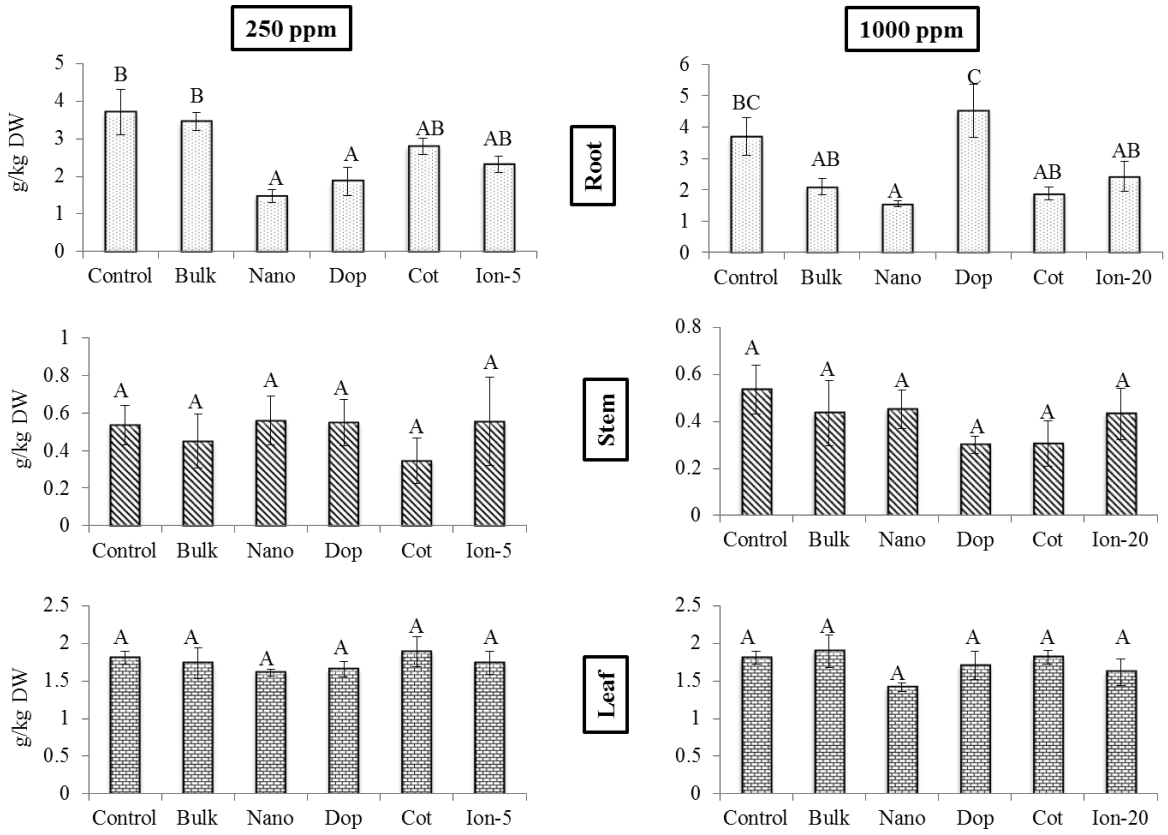


**Fig. 4.7** Silicon bioaccumulation in root, stem, and leaf tissues. Bars with same letters represent no statistical significance at $p \leq 0.05$.

### 4.3.4 Chlorophyll and carotenoid in leaf

Amount of Chl-*a* was significantly increased at 250 mg $kg^{-1}$doped treatment (4.5x) and in all the treatments of 1000 mg $kg^{-1}$ [bulk (3.2x), bare (2.7x), doped (3.6x), coted (2.5x), and ionic (2.4x)] compared to control (Figure 4.8). However, there was no difference in the amount of chlorophyll-*b* (Chl-*b*) (Figure 4.9). The total carotenoid was increased significantly at 250 mg $kg^{-1}$ to 10x in doped and 7x times in ionic treatment (Figure 4.10). The increase was 7.6x in bulk and 8.6x in case of doped NPs at 1000 mg $kg^{-1}$ treatments. Unchanged (Chl *b*) or increased

(Chl *a*) content may signify no toxicity effects of different NPs at that particular growth condition.

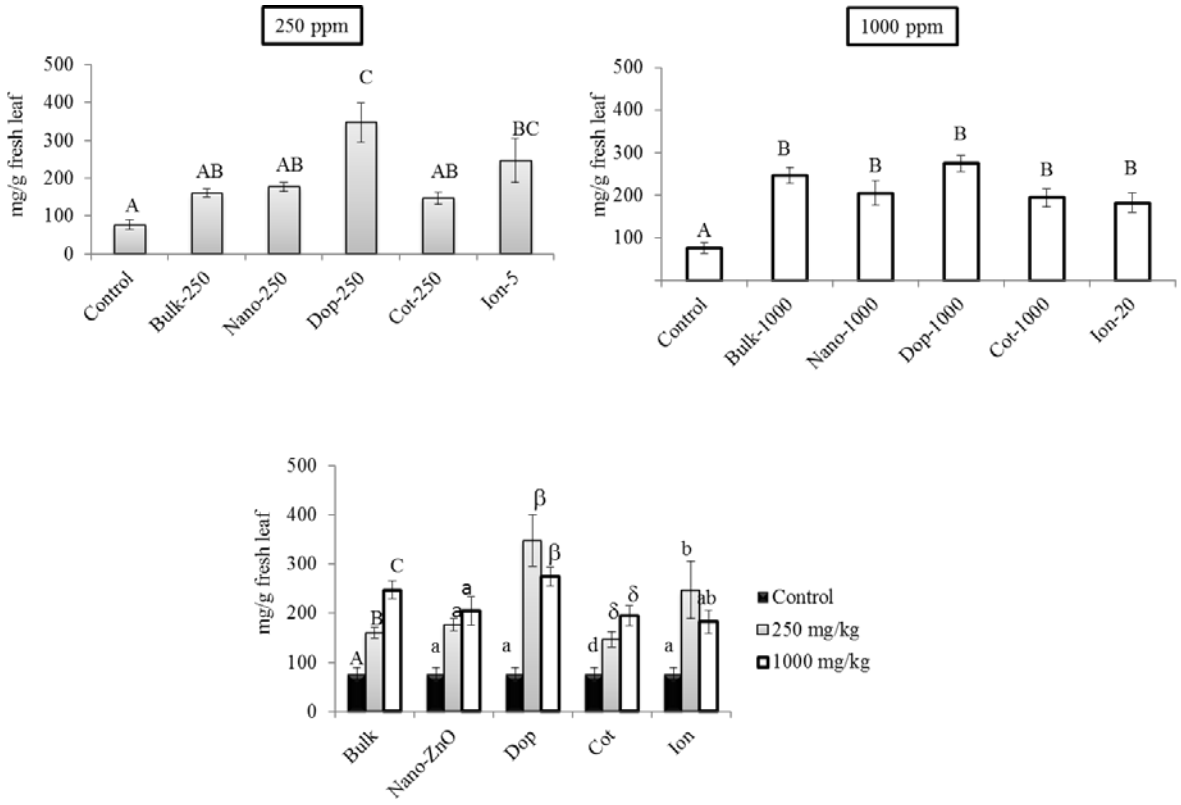


**Fig. 4.8** Chlorophyll-*a* concentrations in leaf tissues. Bars with same letters/symbols represent no statistical significance at $p \leq 0.05$.

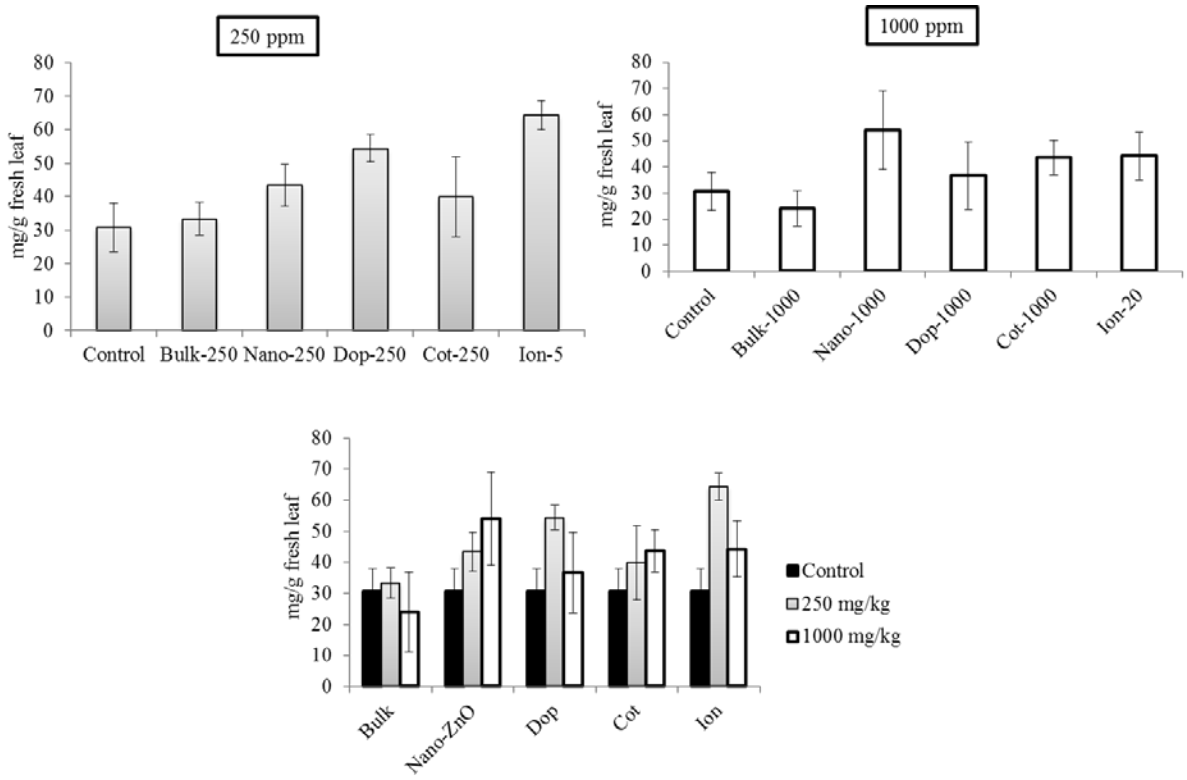


**Fig. 4.9** Chlorophyll-*b* concentrations in leaf tissues. Bars with no letters/symbols represent no statistical significance at $p \leq 0.05$.

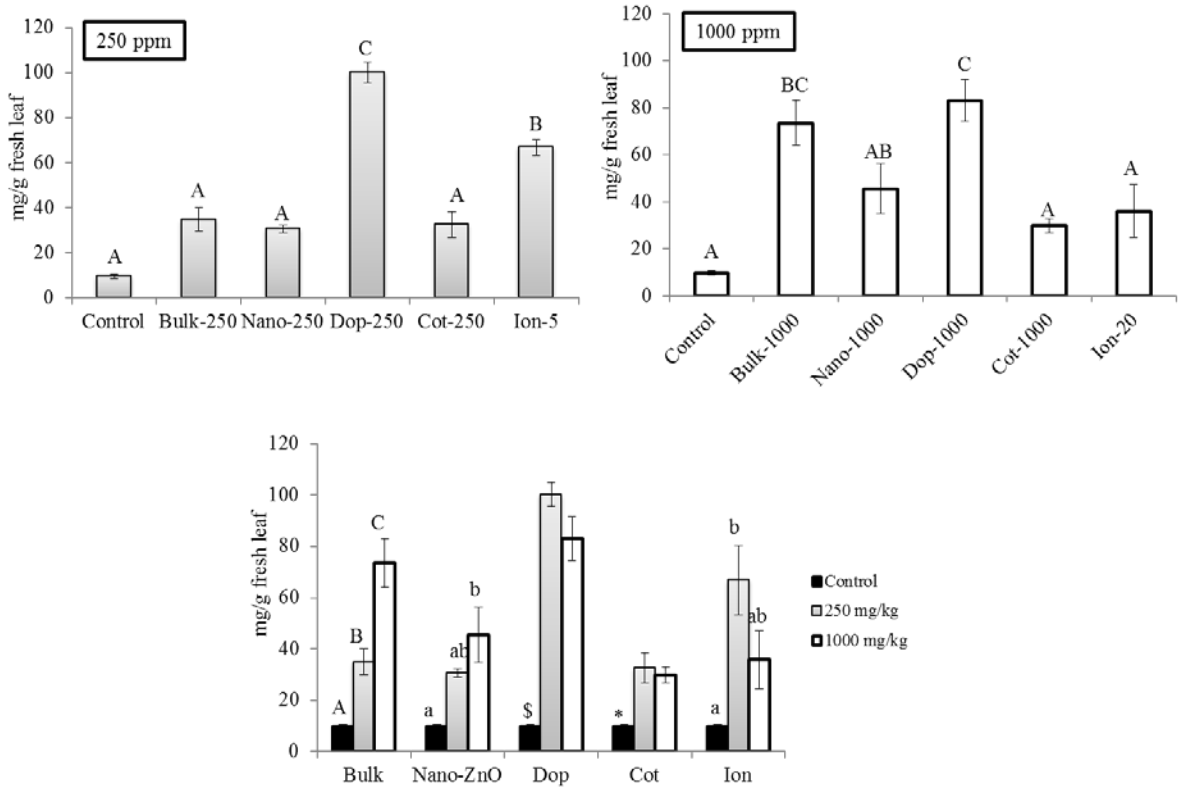


**Fig. 4.10** Total carotenoid concentrations in leaf tissues. Bars with same or no letters/symbols represent no statistical significance at $p \leq 0.05$.

### 4.3.5 Effects of NPs on green pea seed quality

#### 4.3.5.1 Pod length, pod weight, and number of seeds per pod

The NP treatments also altered the pod characteristics of green pea. The pod lengths, pod weights, and number of seeds per pod did not change among treatments with the exception of doped 250 mg $kg^{-1}$ treatment where the number of seeds per pod decreased by 33% compared to that of bare ZnO NP treatment. This might be attributed to higher toxic effect of doped NPs compared to other ZnO NPs.

#### 4.3.5.2 Mineral concentration

Green pea seeds are good source of K, Mg, Cu, Mn, and P (*44*). Therefore, these nutrients were quantified in green pea seeds from all the treatments. In seed (pea), zinc accumulation at 1000

mg $kg^{-1}$ was increased in all the treatments ranging from ~2x to 2.5x, compared to control, except for the bulk and ionic treatment (Fig. 4.11A).

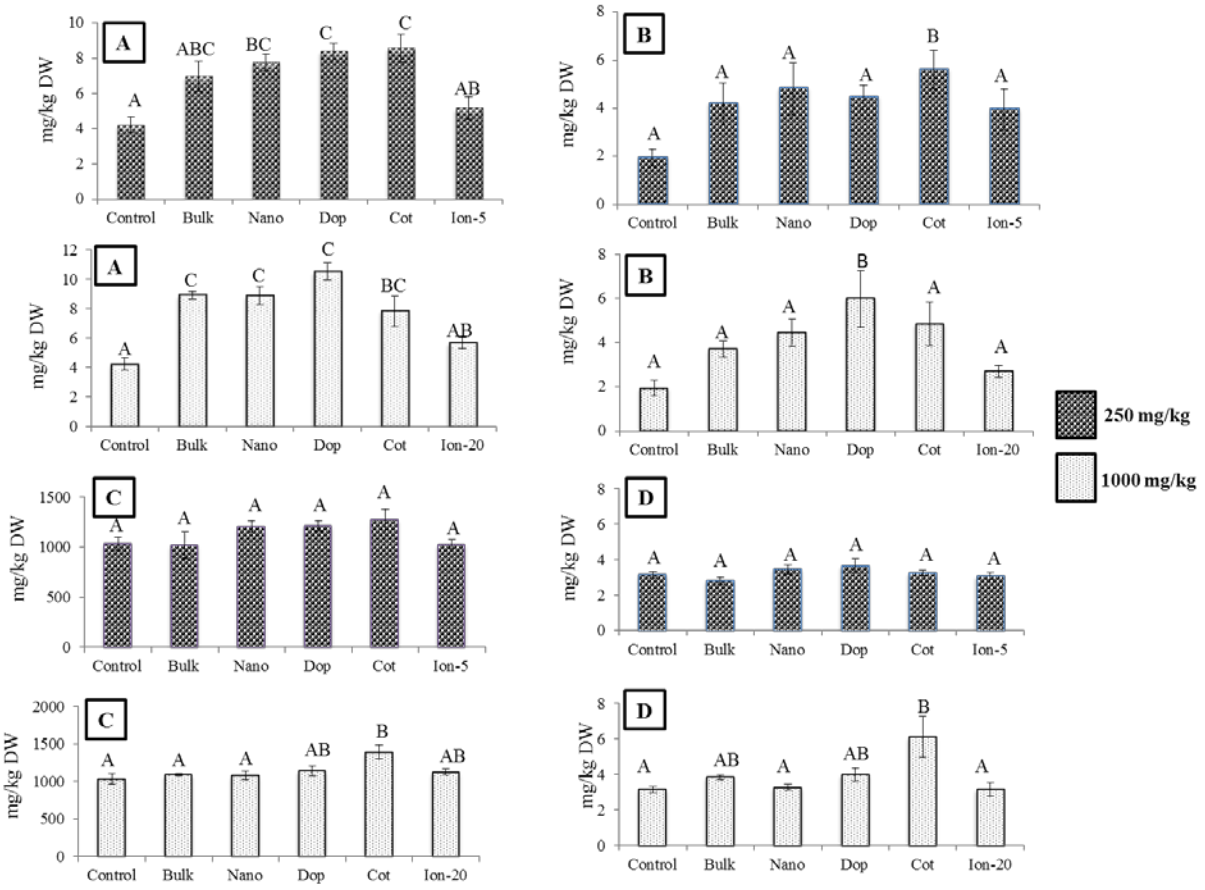


**Fig. 4.11** (A) Zinc (B) iron (C) phosphorus (D) manganese bioaccumulation in seeds at 250 and 1000 mg/kg treatments. Bars with same letters represent no statistical significance at $p \leq 0.05$.

A threefold (3x) increase in aluminum, silicon, and iron content was recorded in all treatments, except 250 and 1000 mg $kg^{-1}$ coated treatment. However, copper, magnesium, phosphorus (except 1000 mg $kg^{-1}$ coated treatment increased 35%), manganese (except 1000 mg $kg^{-1}$ coated treatment increased by 2x), potassium bioaccumulation did not change with

changing treatments (Fig. 4.11B-D). Our results indicate that NP treatments has less affect in the nutritional quality of green pea seeds however, coated and doped treatments are changing iron, phosphorus, and manganese bio-accumulation at higher concentrations (1000 mg/kg). These nutrients are considered as essential and, hence, the coated and doped NPs are affecting the seed quality of green pea.

#### 4.3.5.3 Protein and carbohydrate profiles

The amount of acid-soluble (glutelin)**,** salt-soluble (globulin), water-soluble (albumin), and alcohol-soluble (prolamin) protein fractions remained unaltered at all the treatments (Fig. 4.12). There was a decrease in glutelin amount (50%) were recorded at 1000 mg/kg doped treatment compared to control, but the decrease was statistically insignificant. This was may be due to less number of replicates (four) and/or higher standard errors of the measurements. Therefore, the treatments did not affect the proteins contents of green peas in those concentrations. The amount of total sugar, starch, reducing sugars (glucose and fructose), and non-reducing sugar (sucrose) also remained unaltered, except with1000 mg/kg doped treatment, where the sucrose content was significantly increased (1.8x) compared to control (Fig. 4.13).

In plants, sugars mainly serve as a source of energy (*52*). In addition, reducing and non-reducing sugars can contribute to the signaling pathways related to stress (*53-55*). Therefore, higher sucrose concentration in green pea at 1000 mg/kg doped treatment not only indicating the effect on seed quality but also possible indicator of stress (*53-55*).

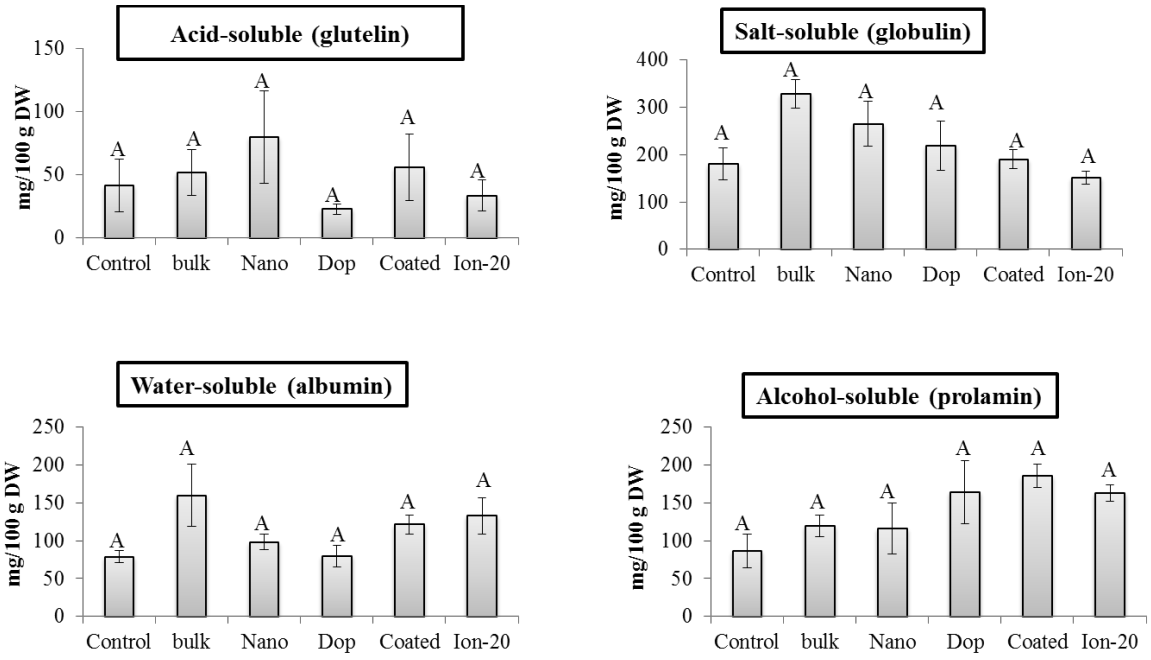


**Fig. 4.12** Amount of different protein fractions in seed. Bars with same letters represent no statistical significance at $p \leq 0.05$.

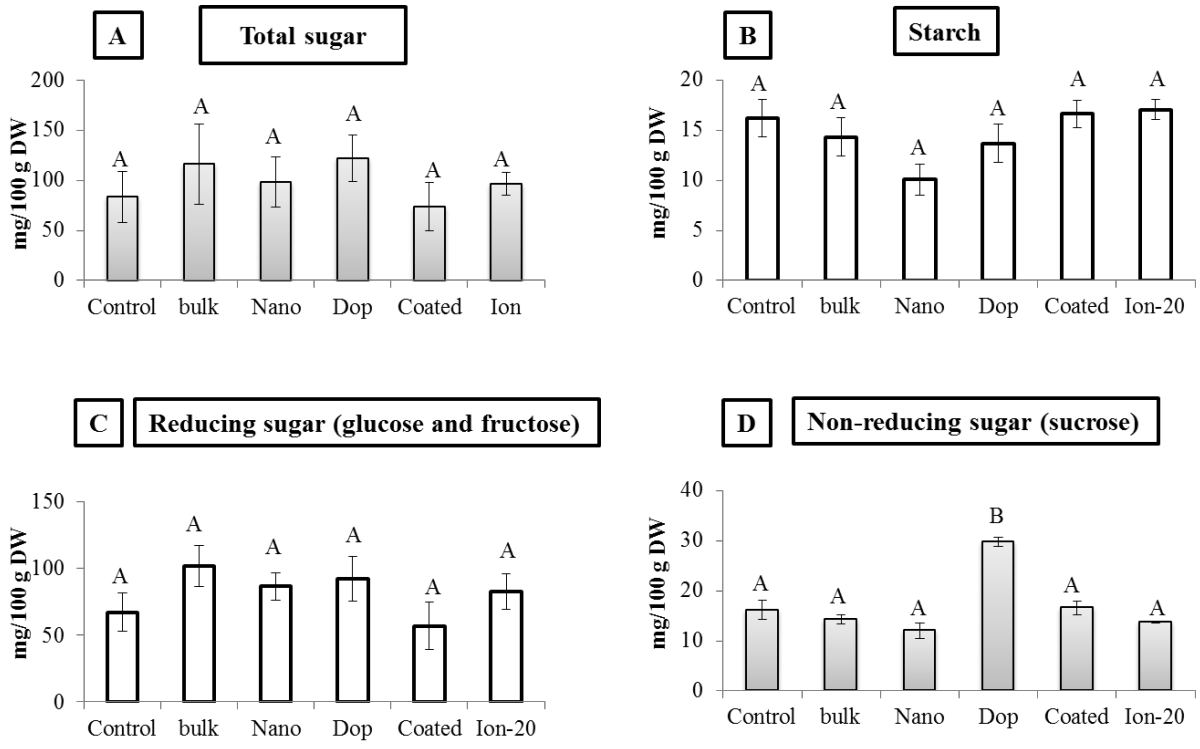


**Fig. 4.13** Carbohydrate profile in seed. (A) Total sugar, (B) Starch, (C) reducing sugar, and (D) non-reducing sugar contents in seed. Bars with same letters represent no statistical significance at $p \leq 0.05$.

In summary, our study indicated the comparative phyto-toxicity of bare-ZnO NPs, $Al_2O_2$@ZnO, and ZnO@KH550 NPs on green pea plants in terms of biomass, Zn bio-accumulation, changes in photosynthetic pigment contents, along with the changes in seed characteristics and seeds' quality. The results confirmed that in spite of larger size (15 nm) of the doped ZnO NPs, these NP exerted more effects than that of the bare ZnO NPs (10 nm) in green pea plants. Additionally, the green pea seed was affected only by the doped NPs at 1000 mg/kg as per non-reducing sugar (sucrose) content was concerned. Considering our Phase III results, $Al_2O_3$@ZnO NP treatment at highest concentration was found to be more toxic to green pea,

compared to all other different NP treatments. Therefore, in short term, higher level of exposure might affect the seed quality of green peas and hence altering the nutritional profile of green pea seed.

# Chapter 5

## Uptake and toxicity of coated and uncoated silver nanoparticles to green peas, corn, and zucchini


### Abstract

In recent years, silver nanoparticles (AgNPs) are being widely used in consumer products. Due to widespread use, these NPs are being released into the environment and may impose adverse effects on living organisms. In this study, we have investigated the effects of short-term exposure (20 days) of coated and uncoated AgNPs on green peas (*Pisum sativum* L.), zucchini (*Cucurbita pepo*), and corn (*Zea mays*). Plants were treated with 500, 1000, and 2000 mg/kg bare AgNPs (20 nm), two different types of PVP coated AgNPs (0.2 wt% Ag-PVP-L, 30-50 nm and 0.2 wt% Ag-PVP-Sm, 20 nm), bulk silver, and silver sulfate (Ag-ion, at 5, 10, and 20 mg $kg^{-1}$) in soil. Upon harvest, total Ag uptake was measured and different physiological parameters were monitored. In green peas, fresh weight was significantly reduced in all the treatments (except Ag-ion) keeping the dry-weight unaltered, compared to control. Silver uptake increased, at all the treatments, in a concentration dependent manner (except Ag-ion) in green peas. Shoot accumulated higher amount of Ag only at 2000 mg/kg treatments with an exception of Ag-ion. Coated Ag NPs affected the amount of Chl-*a* and Chl-*b* in green pea leaves. The bare AgNPs showed reduction in carotenoid concentrations at 500 and 1000 mg/kg treatments. In zucchini, fresh weight decreased in all the NP treatments (except 500 mg/kg Ag-PVP-S and AgNP 2000 mg kg−1) treatments compared to control, keeping the dry weight unaffected. Root uptake increased in a concentration dependent manner at 500 and 1000 mg/kg. Shoot uptake increased only at 500 mg kg−1 Ag-PVP-L treatment. Chlorophyll and carotenoid amounts were mostly unaffected by Ag-PVP-S treatments. Fresh and dry weights remain unaffected by all the

treatments in corn. Root uptake of silver was increased (15x to 26x) considerably. However, the shoot uptake increased only at Ag-ion-10 treatment. Ionic treatments showed comparable silver uptake with that of all the NP-treatments. Chlorophyll and carotenoid amounts were not affected by any of the treatments (except 1000 and 2000 mg/kg Ag-PVP-S treatment). The results indicate that in dicots, silver uptake was higher with PVP-AgNP-Sm treatments compared to that of bare AgNPs and PVP-AgNP-L. Lower silver uptake in corn zucchini compared to green peas might be due to the adverse effects of silver in corn and zucchini roots compared to that of green peas. To confirm the differential toxic effects of Ag NPS on different plants and corresponding toxic mechanisms, further investigation is needed

**Keywords:** coated and uncoated AgNPs, silver uptake, chlorophyll *a*, chlorophyll *b*.

## 5.1 Introduction

Silver nanoaprticles (AgNPs) are being used in different industries, e.g., healthcare, textile, and agriculture, among others, mainly due to its antibacterial and photochemical properties (*1-5*). Silver is one of the most toxic metals known. However, the mechanism of toxicity into terrestrial plants is not being unveiled (*6*). It has been reported that AgNP exposure can cause oxidative stress, reduce photosynthesis, and chlorophyll content in aquatic plants by releasing silver ions (*6-7*). Toxicological studies of AgNPs are mostly directed to bacteria (*9-10*), algae (*7, 11, 16*), fish (*12*), and human cell lines (*8, 13*). The reported toxicity was mostly due to the released silver ions in solution or test media (*9-14*, *17*).

Choi et al. studied a size dependent toxicity of AgNPs on nitrifying bacteria (*10*) and reported that at equivalent silver concentrations, smaller AgNPs (5 nm) were more toxic to bacteria than larger AgNPs or Ag-ions (*10*). Comparative toxicity of ionic Ag and AgNPs were studied on alga *Chlamydomonas reinhardtii* and the grazing crustacean *Daphnia magna* by McTeer et al. (*14*). The authors reported that the feeding of *D. magna* was significantly reduced when fed with silver nitrate treated algae than that of AgNP. This is due to the higher silver concentration inside the algae treated with silver nitrate compared to that of AgNP. In this study, algae treated with silver nitrate showed more toxicity to *D. magna* than algae treated with AgNPs (*14*). Navarro et al. also reported higher toxicity of silver ions compared to its nano form in a freshwater alga (*Chlamydomonas reinhardtii*) (*11*). In addition, Gubbins et al. studied the phytotoxicity of silver nanoparticles to *Lemna minor* L. and inhibition of plant growth was evident with exposure of small (~20 nm) and larger (~100 nm) AgNPs at very low concentrations (5 μg $L^{-1}$). The authors also reported acute phytotoxicity with a longer exposure time (*15*).

Limited studies have been reported regarding the interactions of AgNP with higher plants. For example, Kumari et al. investigated the genotoxic and cytotoxic effects of AgNPs (<100 nm) on *Allium cepa* using its root-tip cells (*16*). Authors found that AgNPs can enter into the roots causing stickiness and disturbed metaphase, leading towards cell death (*16*). Stampoulis et al. also reported the phytotoxic effects of Ag NPs on crop plant *Cucurbita pepo* (zucchini) (*17*) in hydroponic solution. The study reported that Ag NPs exposure resulted in 57% and 41% reduction in plant biomass and transpiration, at 500 and 1000 mg/L treatments, respectively, as compared to controls or to plants exposed to bulk Ag (*17*). The resulted phytotoxicity was reported due to the released ion in the growth medium. Recently, Lee et al. also investigated the phytotoxicity of silver nanoparticles (AgNPs) on the important crop plants, *Phaseolus radiatus* and *Sorghum bicolor* in soil and agar medium where authors reported a differential phytotoxicity of Ag NPs depending on the growth medium (*18*). Authors reported that the growth rate of *P. radiatus* was not affected in soil by impediment within the concentrations tested. The results indicated a reduced bioavailability of Ag NPs in soil, and the dissolved silver ion effect also differed in the soil as compared to the agar (*18*). Therefore, the phytotoxicity of Ag NPs is dependent on the growth medium and/or size and chemical coating of the NPs (*15*-*18*). On a different side, De La Torre-Roche et al. studied the effects of nano, bulk, and ionic forms of silver on the bioaccumulation of dichlorodiphenyldichloroethylene (p,p′-DDE; DDT metabolite) in soybean (*Glycine max* L.) and zucchini (*19*). Results showed that silver could significantly affect the uptake and translocation of DDE, one of the common agricultural contaminants (*19*). Therefore, current literatures not only report the phytotoxic effect of Ag NPs but also evidenced that the toxicity of other contaminates can be triggered/enhanced by the presence of Ag NPs in the environment.

To the best of author's knowledge, there is no report on size dependent comparative phytotoxic studies of Ag NPs and their bulk and ionic counterpart on food crops, namely; green pea, zucchini, and corns. Therefore, research exploring this gap will be useful for the scientific community to understand the food crop-NPs interactions and the identification of potential toxic species.

The present study was aimed at inspecting the phytotoxic effects of bare AgNP, Ag-PVP-L, and Ag-PVP-Sm on three different plants, green peas, zucchini, and corn. Plants were treated with 500, 1000, and 2000 mg/kg bare-AgNPs (20 nm), two different types of PVP coated AgNPs (0.2 wt% Ag-PVP-L, 30-50 nm and 0.2 wt% Ag-PVP-Sm, 20 nm) in soil. To differentiate the phytotoxic effects of different Ag NPs versus micron size particles or dissolve ionic species, both bulk silver, and silver sulfate (Ag-ion, at 5, 10, and 20 mg $kg^{-1}$) were used in soil. Upon harvest, total Ag uptake was measured and different physiological parameters were monitored. Bioaccumulation of Ag in different parts of plants was determined through ICP-MS. Plants' photosynthetic pigments: chlorophyll and carotenoid content were measured spectro-photometrically. The results indicate that the size and coating on Ag NPs directly affect the uptake and other physiological parameters in plants.

## 5.2 Materials and Methods

### 5.2.1 Exposure assay

50 g soil and 20 ml vermiculite were taken in 125 ml glass jars (Fisher Scientific, Pittsburgh PA). AgNPs (20 nm), PVP coated AgNPs (0.2 wt% Ag-PVP-L, 30-50 nm and 0.2 wt% Ag-PVP-Sm, 20 nm) NPs (US Nano), bulk (Stream Chemicals) and silver sulfate (Stream Chemicals) were added solid to achieve 500, 1000, and 2000 mg/kg concentrations. The jars were capped and vigorously shaken to achieve homogeneity. Seeds were germinated separately

in organic matter enriched soil. Seedlings were transferred (one plant per jar) into the jars, amended with 20 ml 25% Hogland nutrient solution and 20 ml tap water. Each treatment was replicated five times. Jars were transferred in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) with 14 h photoperiod, 25/20 °C day/night temperature, 65% relative humidity, and 340 μmol $m^{-2}$ $s^{-1}$ light intensity. Plants were allowed to grow for 25 days and watered with regular tap water as required.

### 5.2.2 Silver uptake by the plants

Plants were harvested after 25 days treatment of nano, bulk, and ionic silver in soil. Plants were thoroughly washed with tap water for ~5 min and rinsed with 2% $HNO_3$ solution, followed by rinsing with DI water three times (*20*). Roots, stems, and leaves were separated and dried at 70 °C for 24 h prior to digestion. Dry samples were digested in a microwave acceleration reaction system (CEM MARSx, Mathews, NC) following the USEPA 3051 method using plasma pure $HNO_3$ and $H_2O_2$ (1 : 4) (*21*) and analyzed for silver concentration using ICP-MS.

### 5.2.3 Chlorophyll *a*, *b*, and carotenoid measurements in leaves

Razor-blade chopped 100 mg of fresh leaves were taken in a 15 ml tube. Five ml acetone was added, covered with aluminum foil, and vigorously shaken in a horizontal shaker overnight. Samples were taken out and UV-vis was measured with a Perkin Elmer Lambda 14 UV/Vis spectrometer (single-beam mode, Perkin-Elmer, Uberlinger, Germany) at 470, 645, 662 nm. Amount of chlorophyll and carotenoids were calculated using the following equations.

1) $C_a = 11.75\ A_{662} - 2.350\ A_{645}$
2) $C_b = 18.61\ A_{645} - 3.960\ A_{662}$
3) $C_{x+C} = (1000\ A_{470} - 2.270\ C_a - 81.4\ C_b)/230$

Where, $C_a$ and $C_b$ are the concentrations of Chl-*a* and Chl-*b*. From these values, mg Chl per gram leaf was calculated. $C_{x+C}$ is the total carotenoids in μg/g sample (*22*).

### 5.2.4 Statistical analysis

Results were reported as mean of four replicates ± standard error. SPSS Version 19.0 (SPSS, Chicago, IL) was used to perform one-way ANOVA tests followed by Tukey-HSD tests to check the statistical differences between treatment means at $p \leq 0.05$.

## 5.3 Results and Discussion

### 5.3.1 Effect of different Ag NPs/compounds on fresh and dry biomass of plants

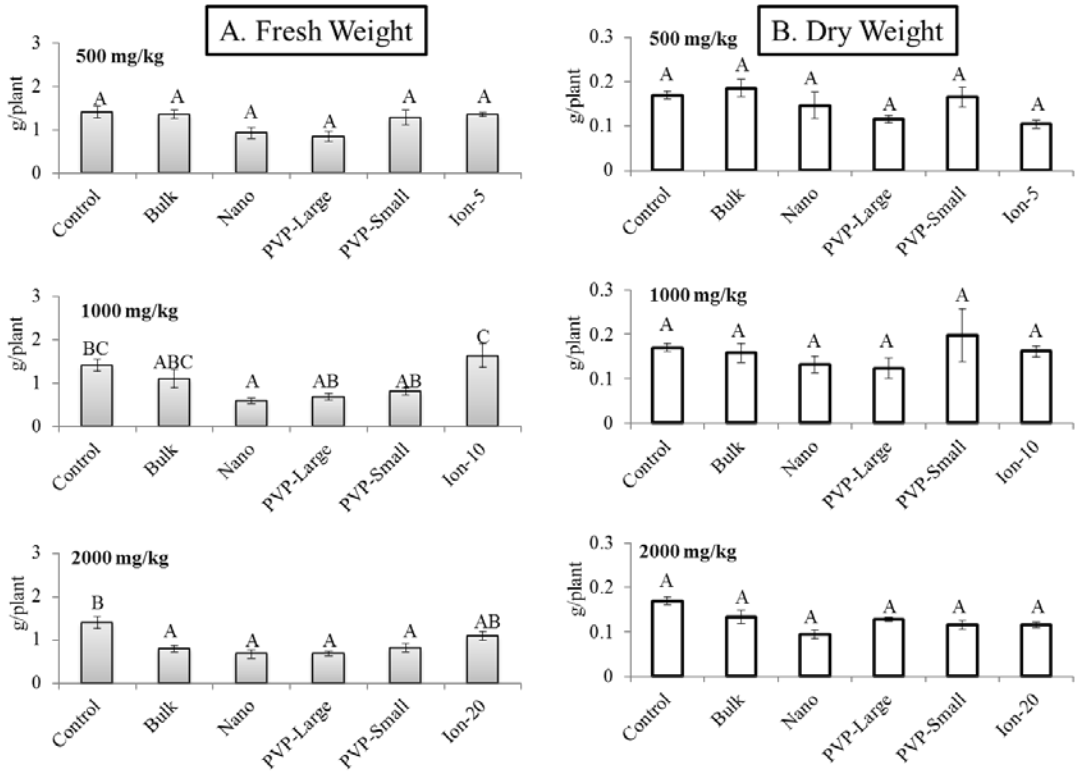


**Fig. 5.1** A) Fresh weight B) dry weight of green pea plants. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

The fresh and dry biomass of pea plants exposed to different Ag NPs are shown in Figure 5.1. Fresh weight was significantly reduced in all the treatments (except Ag-ion) keeping the dry-weight unaltered, compared to control. At 2000 mg/kg treatment of Ag NPs, the fresh weight of green pea plants decreased significantly to 1.75 to 2 times, compare to control (except ionic treatment). In addition, bare-AgNPs at 1000 mg/kg reduced fresh weight by ~3x. However, the dry weights remained unaltered in al the treatments (Fig. 5.1B). A reduced biomass leads to phytotoxicity of Ag NPs (*15*, *17*, *18*).

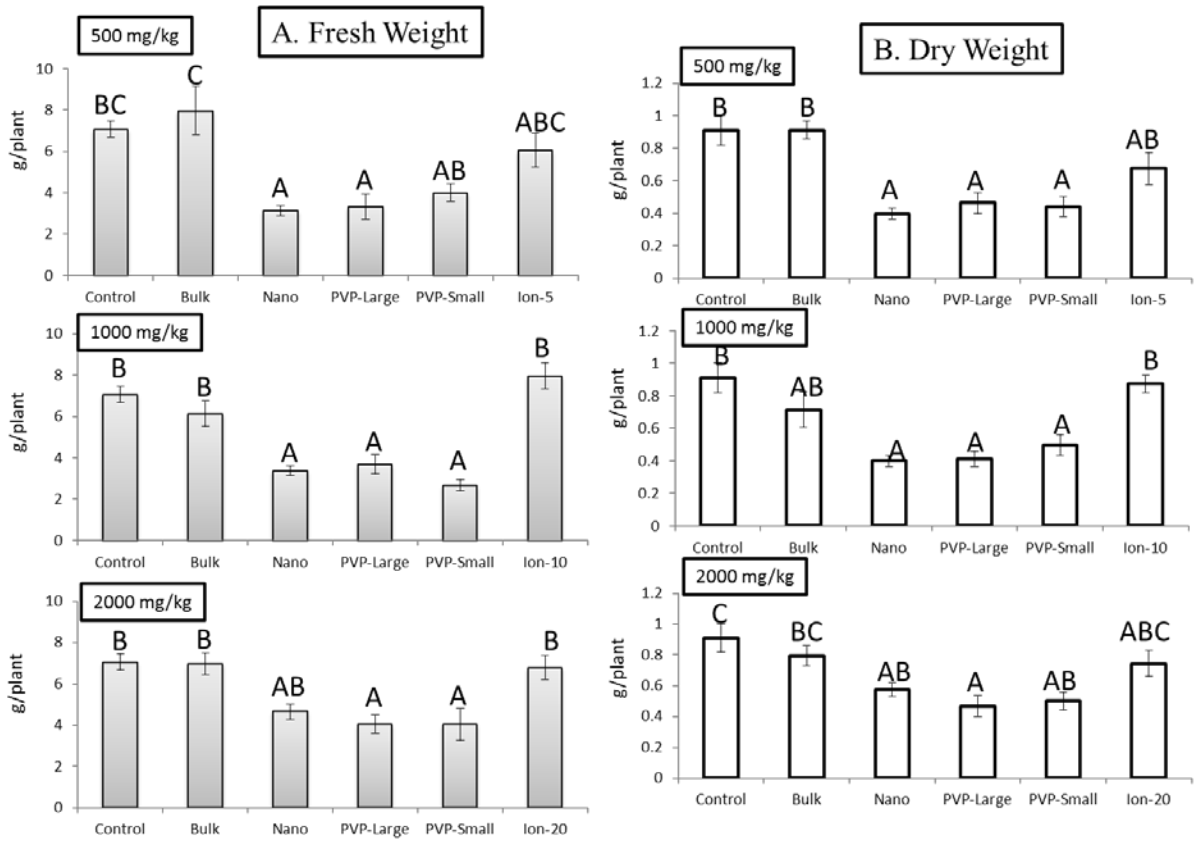


**Fig. 5.2** A) Fresh weight B) dry weight of zucchini plants. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

In zucchini, both the PVP coated NPs showed significant decrease (1.7x to 2x) in fresh weight compared to control (Fig. 5.2A). However, dry weight decreased at 500 and 1000 mg/kg bare AgNP treatments significantly to ~2x(Fig. 5.2B), compared to control. Our results are in accordance with the reported literature of Stampoulis et al. where decreased biomass was evidenced with increased concentration of AgNPs in zucchini (*17*). Corn showed no significant change in fresh and dry weights by any treatments, compared to control (Fig. 5. 3A, B).

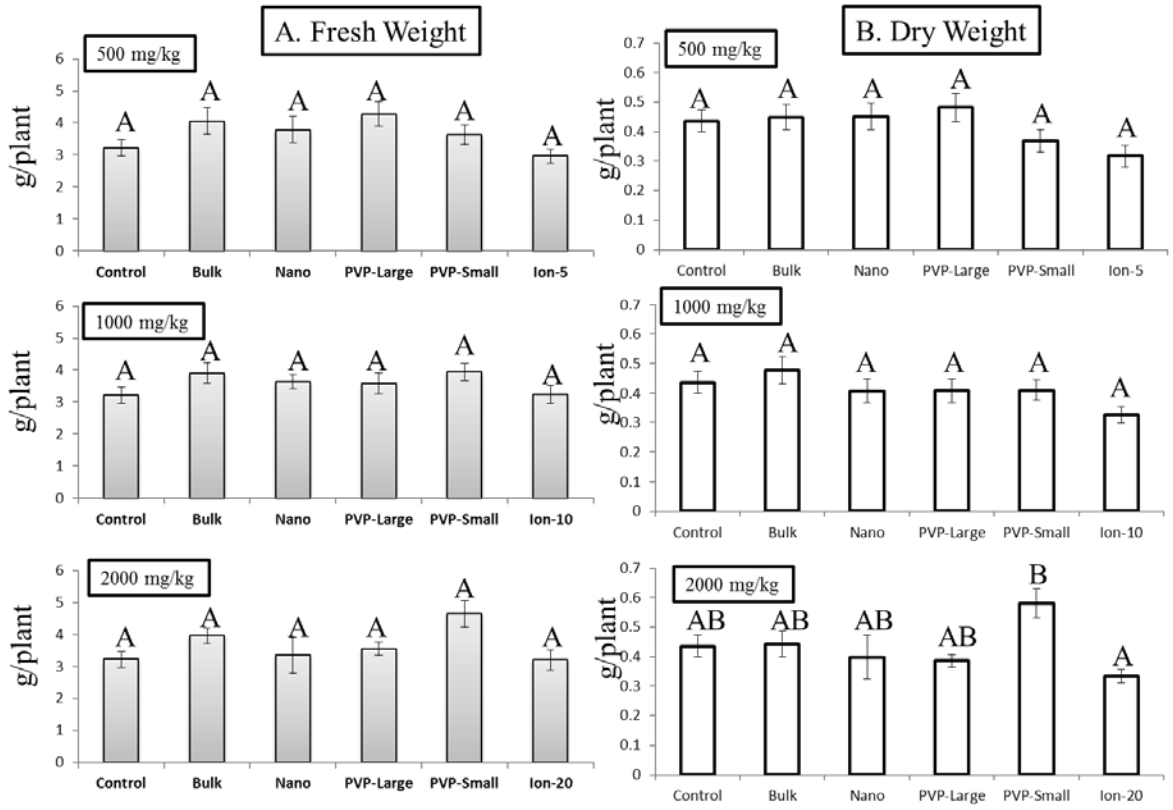


**Fig. 5.3** A) Fresh weight B) dry weight of corn plants. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

There was visual damage to the roots in case of zucchini. This was also evident in literatures (*17*) where root tip damage was found with Ag NPs application. However, no visual damage was observed in case of green peas and corn. This could be attributed to the species dependent differential phytotoxicity of different NPs. Our results indicated that zucchini was affected mostly by silver NPs compared to green peas or corn in terms of biomass reduction.

### 5.3.2 Bioaccumulation of silver

In roots, silver uptake increased in a concentration dependent manner in all the treatments (except the ionic treatment) compared to control.

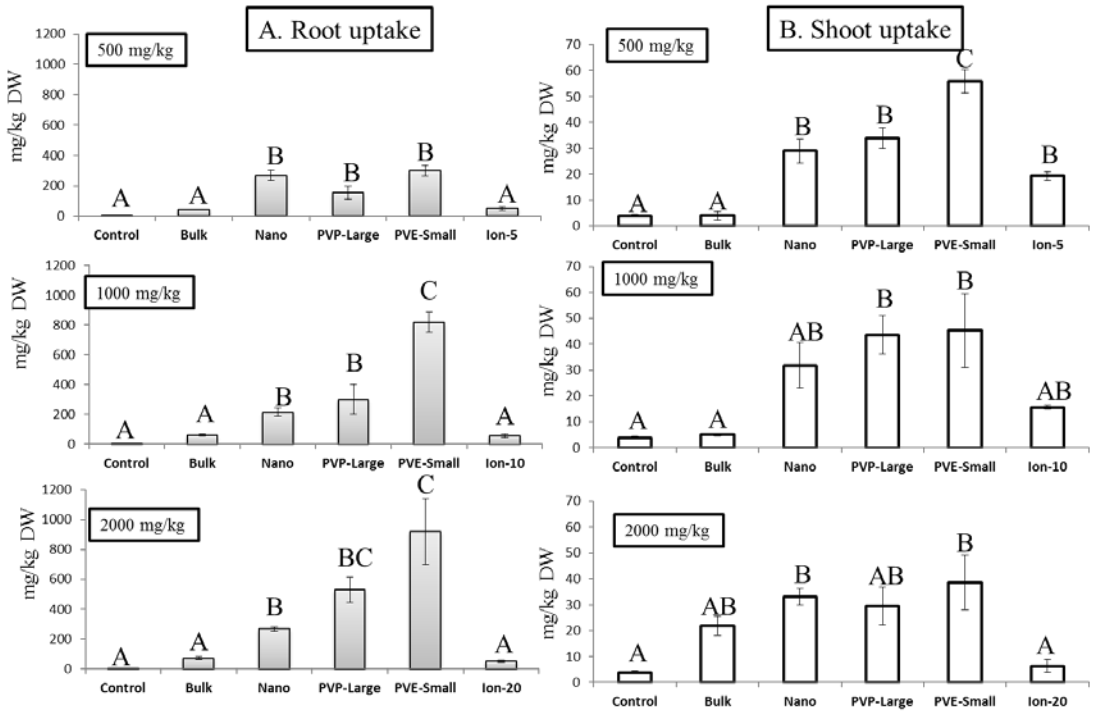


**Fig. 5.4** Silver accumulation in A) roots and B) shoots of green pea plants. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

In all treatments, at all concentrations (except Ag-PVP-L at 2000 mg/kg) root silver uptake increased in the range of 33 to 196 times compared to control (Figure 5.4A) in green peas. The shoot Ag uptake was increased to 7x to 14x range at all the NP treatments (except 2000 mg/kg Ag-PVP-L) (Fig. 5. 4A, B).

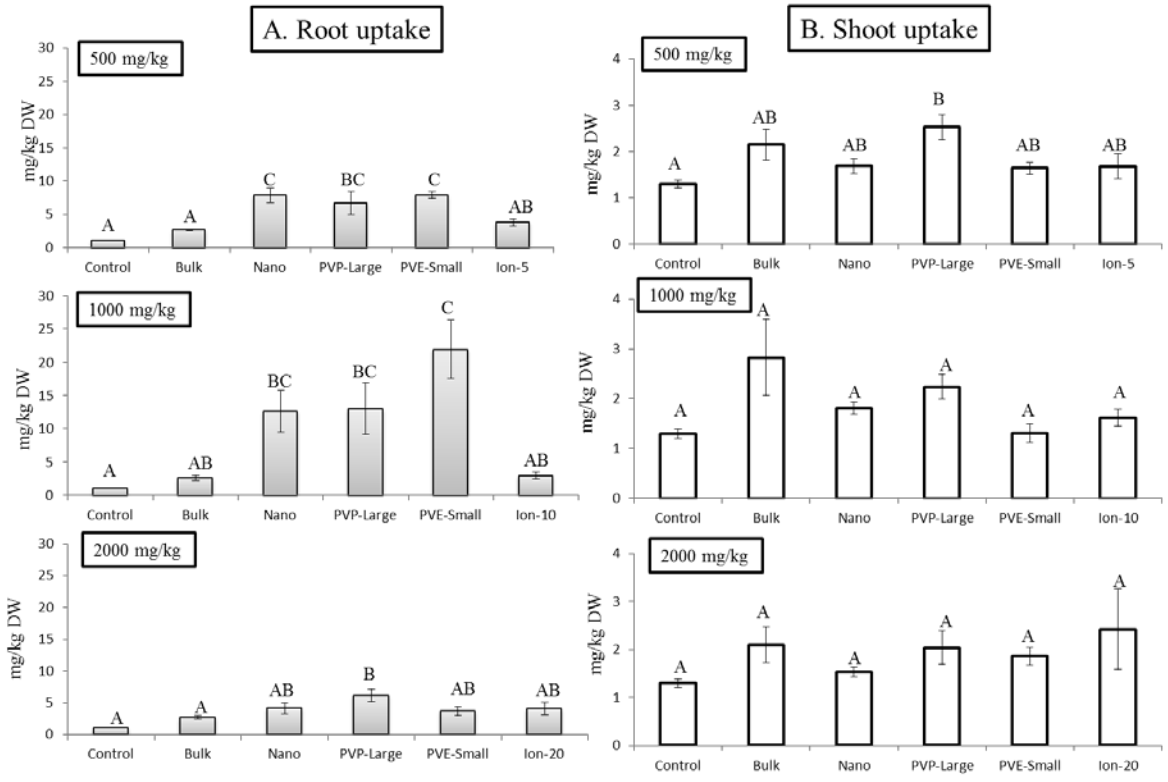


**Fig. 5.5** Silver accumulation in A) roots and B) shoots of zucchini plants. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

In zucchini, silver uptake in root was increased at 500 and 1000 mg/kg treatments for all the NP, compatred to control? (Fig. 5.5A). At 2000 mg/kg, only large coated treatment showed significant increase (6x) in silver uptake compared to control. However, in stem, silver uptake increased significantly (2x) only at 500 mg/kg Ag-PVP-L treatment compared to control (Fig. 5.5B). In corn, at all the treatments, including bulk and ionic showed significant increase in root uptake (15x to 26x) (Fig. 5.6A, B). All these results of increased Ag uptake with increased NP dose followed the previously reported literatures (*15-18*). However, different dose response/uptake was noticed in corn than that of green pea and zuccihini.

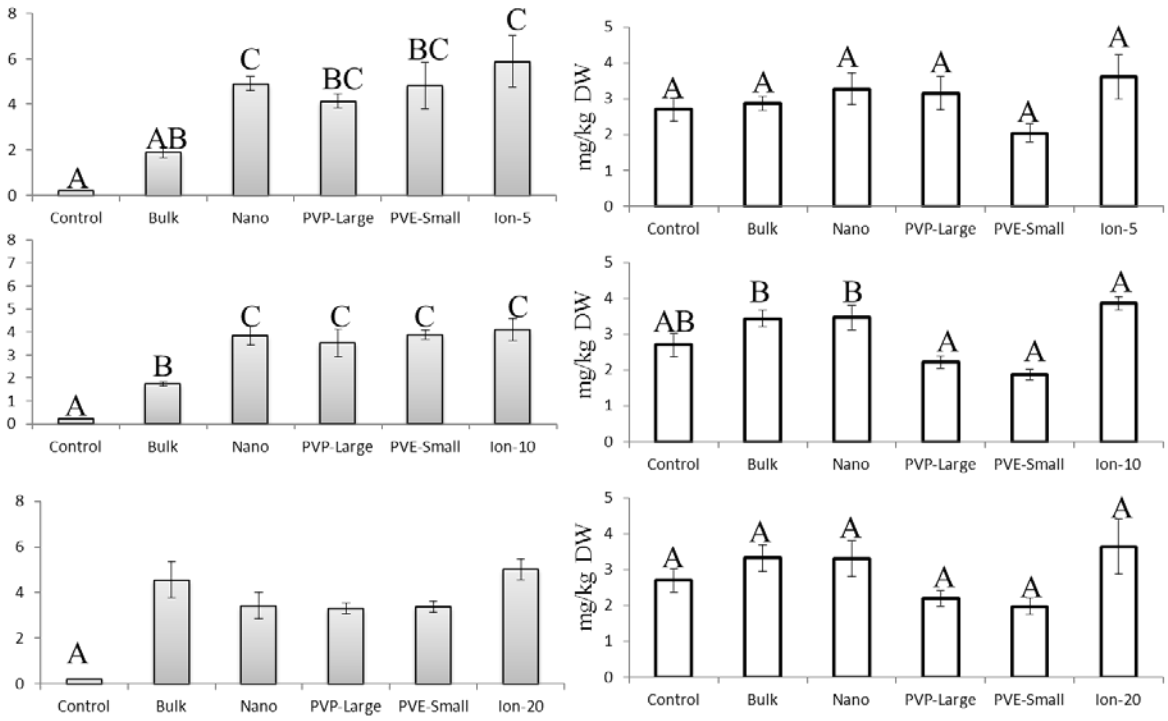


**Fig. 5.6** Silver accumulation in A) roots and B) shoots of corn plants. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

In corn, the amount of silver uptake by the roots was highest in ionic treatments than the NP treatments might be attributed to root tip damage due to NP exposure. Similar studies have been reported by Yin et al. where decreased uptake of Ag was due to root tip damage by silver NP in common grass (*Lolium multiflorum*) (*6*). Therefore, NP treatments might damage the root-caps of corn plants, leading towards less silver absorption in the NP treatments compared to the ionic. However, root-tip imaging needs to be done to confirm this possibility.

### 5.3.3 Effects on chlorophyll-a, b, and total carotenoid

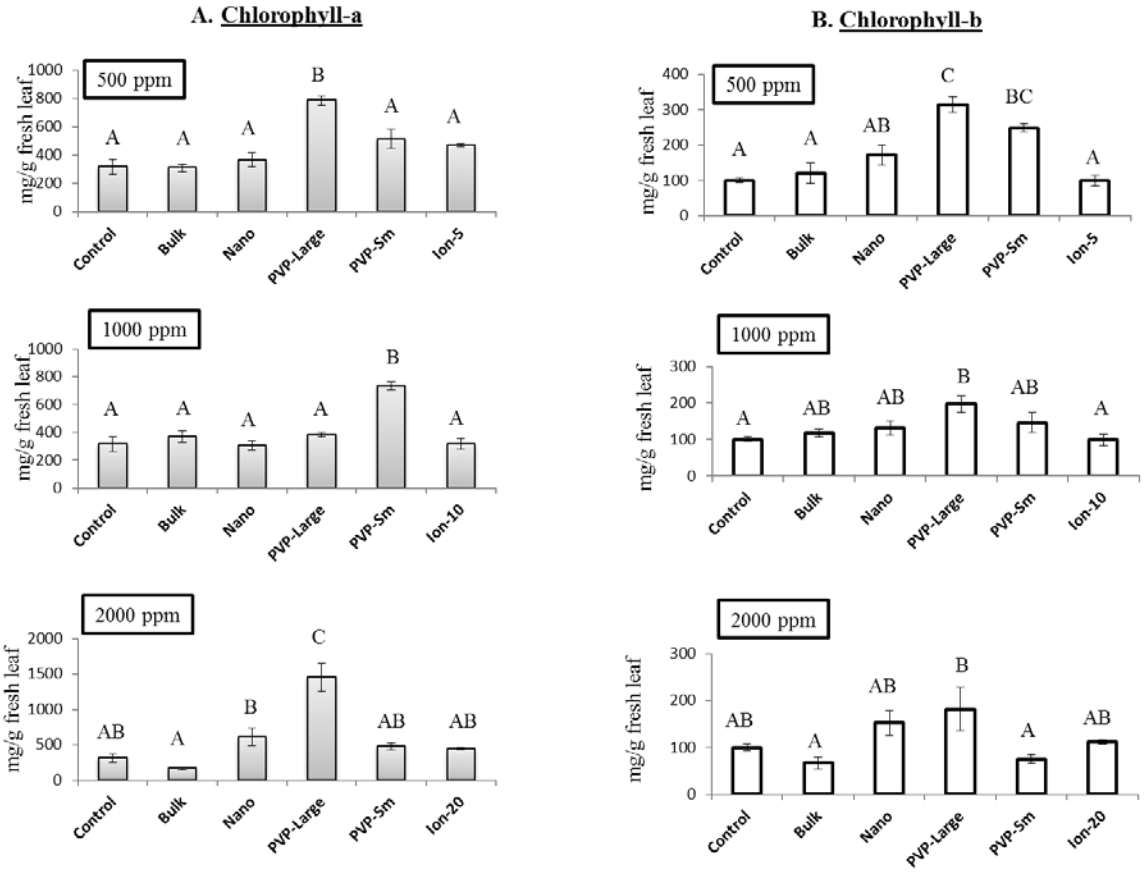


**Fig. 5.7** A) Chl-*a*, B) Chl-*b* concentrations in green peas leaves. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

Chlorophyll and carotenoid amounts were not affected by any of the treatments (except 1000 and 2000 mg/kg Ag-PVP-S treatment) in green peas (Fig. 5.7A, B). However, there was significant increase observed in case of PVP-L NPs at 500 (2.5x) and 2000 (4.6x) mg/kg treatments. Carotenoid amounts also increased at all the concentration in PVP-L treatments (Fig. 5.8). In zucchini, Chl-*a*, *b*, and total carotenoid increased at 1000 mg/kg PVP-Sm (2.25x to 2.6x) and 2000 mg/kg PVP-Sm (2.4x to 3x) treatments (Fig. 5.9A-B, 5.10). No significant difference in Chl-*a*, *b*, and carotenoid concentrations were observed in corn (Fig. 5.11).

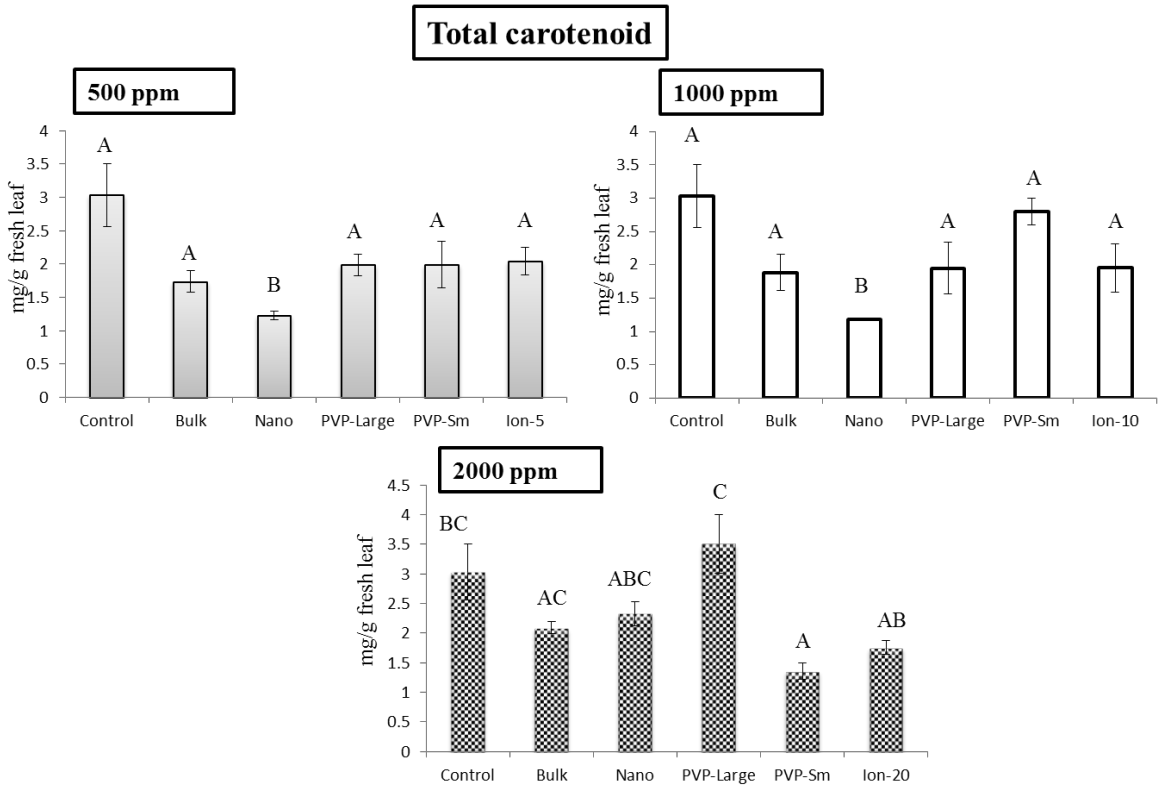


**Fig. 5.8** Total carotenoid in green peas leaves. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

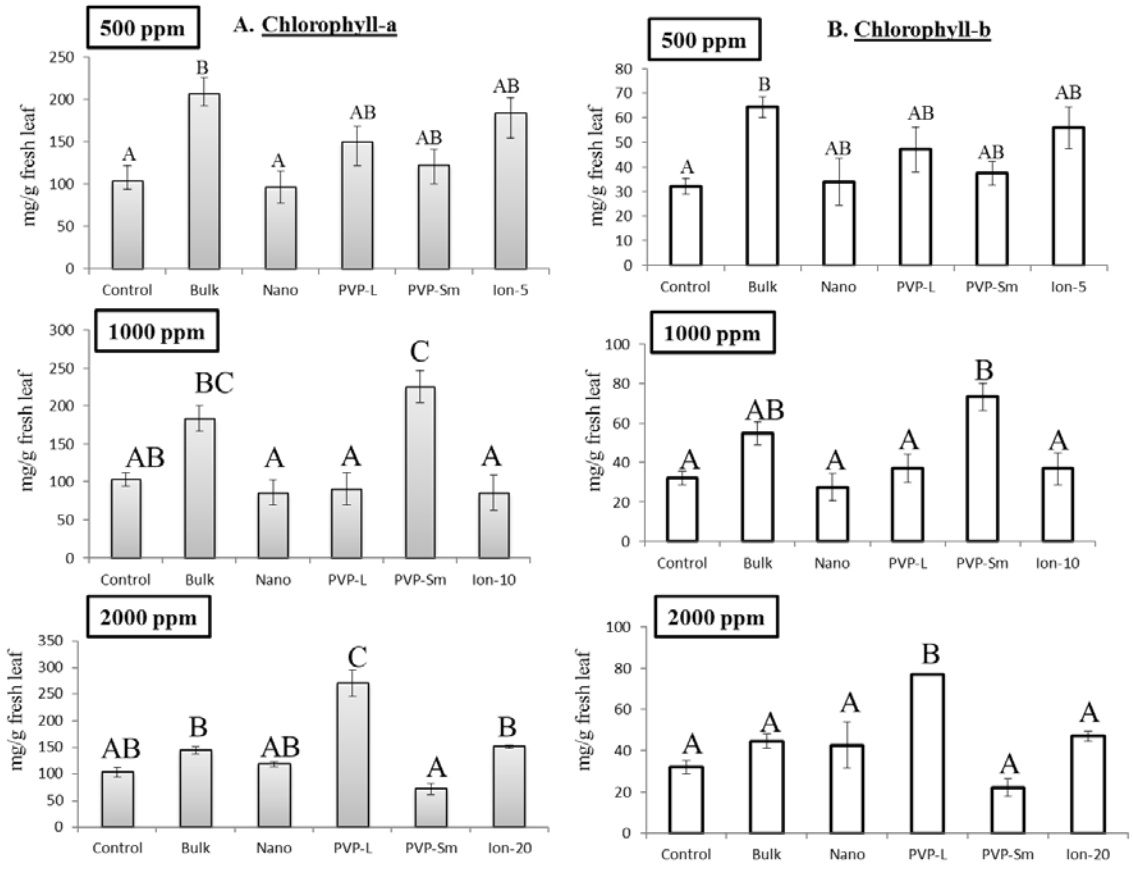


**Fig. 5.9** A) Chl-*a*, B) Chl-*b* concentrations in zucchini leaves. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

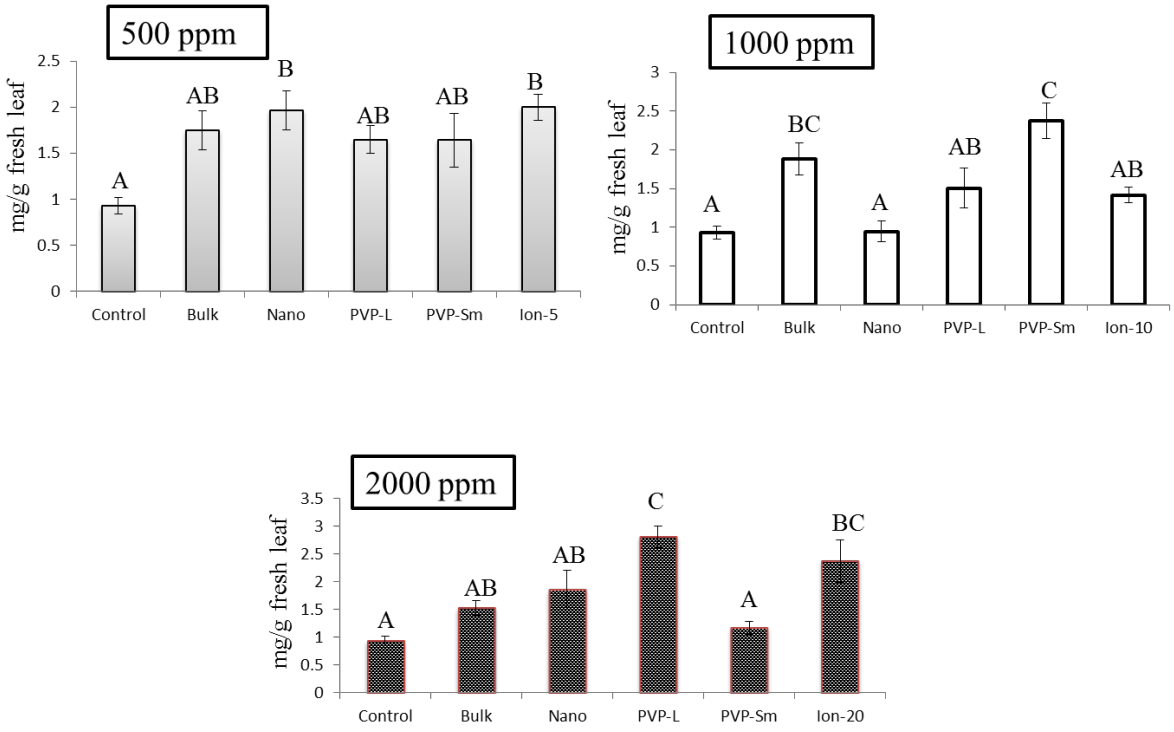


**Fig. 5.10** Total carotenoid in zucchini leaves. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

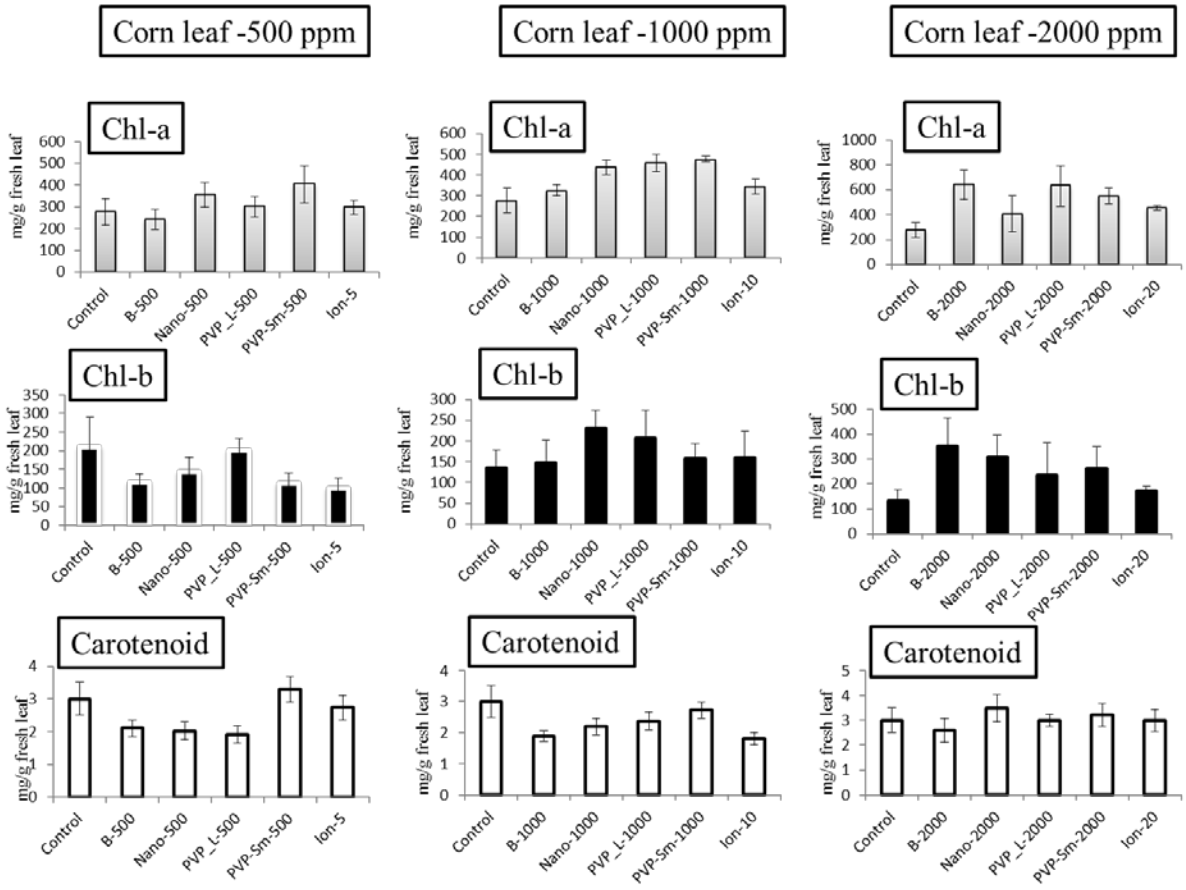


**Fig. 5.11** Chl-*a*, Chl-*b*, and total carotenoid concentrations in corn leaves. Bars represent mean of five replicates ± standard error. Bars with the same letters show no statistically significant difference at $p \leq 0.05$. Comparisons were made between same tissues of different treatments.

The toxicity effects were different in monocot and dicot plants in respect to their uptake, biomass, and photosynthetic pigment content. Toxicological effects of different NPs were found to be significantly different even in similar types of plants (for both the dicots, green peas and zucchini). Results indicate that coated NPs might have some positive impact in green peas, whereas, visual sign of root damage was observed in zucchini. Results established that the phytotoxicity tests based on uptake, biomass, and quantification of photosynthetic pigments might not be enough to assessing phytotoxicity of NPs in these plant species.

# Chapter 6

## General Conclusions

In this study, we have investigated the phyto-toxicological effects of different bare and modified ZnO and AgNPs on green pea, corn, and zucchini plants. Experiments with green pea were performed at two growth stages. In the first experiment, plants were treated with 0, 125, 250, and 500 mg/kg of NPs grown for 25 days in soil. The results demonstrated that, when grown in organic matter enriched soil treated with ZnO NPs, green pea plants accumulate Zn in roots in a concentration-dependent manner. However, less Zn accumulation was observed in bulk ZnO treated plants. ZnO NPs increased root elongation, whereas, the bulk treatments showed both root and stem elongation. The uptake data showed that green pea plant is able to translocate Zn to the aboveground plant parts, for NP treatments, but the translocation was insignificant in case of bulk treatments. The high Zn accumulation, mainly in leaves, induces significant accumulation of $H_2O_2$, and the activity of the stress enzymes CAT and APOX were reduced significantly. But, the bulk treatments showed no effect in $H_2O_2$ and lipid peroxidation. Significant reduction in chlorophyll content and increased lipid peroxidation in plants treated with 500 mg/kg of the ZnO NPs were recorded after 25 days. Our results indicate that ZnO NPs induced more toxicity/stress compared to bulk ZnO for green pea plants in measured traits. However, in Phase II, the plants were exposed to Fe@ZnO NPs in three different concentrations showed diiferent toxic symptoms than that of Zn NPs. Results indicates no visible signs of toxicity including necrosis, stunting, chlorosis or wilting in plants treated with Fe@ZnO in similar growth conditions. However, Fe@ZnO NPs affected different physiological and biochemical parameters in terms of plant growth, chlorophyll content, ROS ($H_2O_2$) production, and antioxidative enzyme activity. We observed that Fe@ZnO is less toxic than ZnO NPs in

green peas grown under the same conditions in terms of relative chlorophyll content and ROS production. Key findings of this work support the fact that greater hydrodynamic diameter and/or lesser dissolution from Fe@ZnO NPs make it less available for green pea plants, and hence lesser phytotoxicity compare to that of bare ZnO NPs. Therefore, iron doping may be considered as an effective alternative approach to reduce the toxicity of ZnO NPs in higher terrestrial plants.

In Phase III, we exposed green pea plants to three different NPs, e.g., bare ZnO NPs (10 nm), 2 wt% alumina doped ($Al_2O_3$@ZnO NPs, 15 nm), and 1 wt% aminopropyltriethoxysilane (KH550 or silane coupling agent) coated (KH550@ZnO NP, 20 nm) NPs along with bulk (ZnO) and ionic Zn (zinc chloride) at 250 and 1000 mg/kg for 65 days. We observed that zinc uptake in root and stem increased in a concentration dependent manner in all types of ZnO NPs. However, treatments with larger doped NP (15 nm) showed higher zinc uptake compared to that of bare (10 nm) NPs. This might be attributed to higher surface potential of the doped ZnO NPs than that of bare-ZnO NPs. Silicon and aluminum concentrations in different plant tissues remained unaffected, which might indicate that most of zinc was being taken up in its ionic form; otherwise, coated and doped NP uptake would have changed the aluminum and silicon concentrations similarly to that of zinc. Nonetheless, very low loading of dopant (2 wt% $Al_2O_3$) and coating (1 wt % KH550) might be another reason for differential uptake of zinc, aluminum, and silicon. At 1000 mg/kg treatments, the amount of Chl-*a* increased significantly in all the treatments. As per chlorophyll concentrations are concerned, it may be concluded that, in ambient conditions (in a greenhouse with constant supply of fertilizer/nutrients), NP treatments showed no toxic effect compared to control. The NP treatments also altered the seed quality in few cases. The effect was insignificant in most cases except higher concentration of doped NPs. At 1000 mg/kg, the green pea seed was only affected in terms of the protein and sugar contents

(total and reducing sugars). Considering our Phase III results, the $Al_2O_3$@ZnO NP treatments was found to be more toxic to green pea compared to all other different NP treatments. The surface (coating) or lattice (doping) modifications may be responsible for differential toxicological behavior of these NPs.

In Phase IV, corn, zucchini and and pea plants were exposed to 500, 1000, and 2000 mg/kg bare AgNPs (20 nm), two different types of PVP coated AgNPs (0.2 wt% Ag-PVP-L, 30-50 nm and 0.2 wt% Ag-PVP-Sm, 20 nm) NPs, bulk silver, and silver sulfate (Ag-ion, at 5, 10, and 20 mg $kg^{-1}$) in soil for 20 days. PVP coated AgNPs showed significant reduction in fresh weights in dicot plants (green peas and zucchini) with no effect in the monocot plant (corn) compared to control. However, the dry weights in both types of plants mostly remained unchanged. In roots of dicots, the extent of silver uptake at 1000 mg/kg treatment found to be the highest at the smallest PVP coated AgNP (up to 174x) treatment. In spite of having the same size with bare-AgNP (20 nm), the extent of silver uptake was higher in the small-coated one. Results suggest that surface coating might play an important role in NP and/or silver uptake. The silver accumulation in uptake is much higher in green peas compared to that of zucchini. This might be due to the fact that, in zucchini, due to observed root damage, the uptake was reduced. But, green pea root systems were unaffected even at the highest concentration. Damage to the central root system might be the reason for less silver uptake. In corn the extent of silver uptake also very less compared to that of green peas. In corn, no visible sign of damage was observed. The toxicity effects were different in monocot and dicot plants in respect to their uptake, biomass, and photosynthetic pigment content. Results indicate that coated Ag-NPs might have some positive impact in green peas (dicot) whereas phytotoxic to corn (monocot).

This entire study concludes the differential phytotoxic effects of different ZnO and Ag NPs on green pea plants and its seed quality (only in ZnO NPs). We also reported the size and surface coating affect differentially in corn and zuccihini grown in soil. Therefore, these results will help to further understand the phytotoxicity of different ZnO/Ag NPs and possible ecotoxicological impacts in the food chain. Future studies must be performed in order to determine the effects of ZnO/Ag NPs on the seed/seed quality of pea plants and also genotoxicity of these NPs.

# Chapter 7


## Acknowledgement

This material is based upon work supported by the National Science Foundation and the Environmental Protection Agency under Cooperative Agreement Number DBI-0830117 and USDA-AFRI (#2011-67006-30181) for fundings. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation or the Environmental Protection Agency. This work has not been subjected to EPA review and no official endorsement should be inferred. The authors also acknowledge the USDA grant number 2011-38422-30835 and the NSF Grant # CHE-0840525. J. L. Gardea-Torresdey acknowledges the Dudley family for the Endowed Research Professorship in Chemistry. The authors also acknowledge Dr. Chuan (River) Xiao biochemistry research laboratory for spectroscopic measurements and Dr. Jose Nunez for his help in the IR studies.

# Chapter 8

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### 8.1 Chapter 1


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**8.5 Chapter 5**

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# Appendix

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# Vita

Arnab Mukherjee was born and raised in Bankura, a district of West Bengal, India. He was the only son of Mr. Apurba Kumar Mukherjee and Sabita Mukherjee. In 2002, Mr. Mukherjee received his Bachelor of Science with Chemistry Hons. (Physics and Mathematics) from the University of Burdwan, India. He received his Master of Science in Environmental Science in 2006 from The University of Burdwan, India. After finishing his MSc degree, Mr. Mukherjee came to USA and joined Western Michigan University, Kalamazoo, MI. In 2011, he received his MA in Science Education degree. In the same year, he joined the PhD program in Environmental Science and Engineering at The University of Texas at El Paso under the supervision of Prof. Jorge Gardea Torresdey.

Mr. Mukherjee received many honors and awards including UTEP Graduate School **Dodson Research Grant**, **Dodson Travel Grant** from the College of Science, UTEP (two times) and ACS Local Chapter Travel Grant. He also received **Phyto-Scholar 2013**[Awarded by the International Phytotechnology Society (IPS) and the National Institute of Environmental Health Sciences (NIEHS)] and **SNO Student Award 2013** (Awarded by the Sustainable Nanotechnology Organization]. In UTEP, Mr. Mukherjee served as the **Chair of the Green Fund Advisory Committee** (Fund > $250k) and a board member of the **President's Advisory Committee on Sustainability**. He served as a member of the committee for The Sustainability Tracking, Assessment & Rating System™ (STARS).

While pursuing his PhD, Mr. Mukherjee worked as a teaching/research assistant for the chemistry department. Mr. Mukherjee has presented his research in many national conference meetings including the American Chemical Society's National Meetings. Additionally, Mr. Mukherjee has published two original research articles as the principle author and two others are

under review (as a co-author). In addition, Mr. Mukherjee has published a book chapter in International Union of Pure and Applied Chemistry (IUPAC), John Wiley and Sons, Hoboken, NY, USA. He got a fulltime post-doctoral scientist position in The Connecticut Agricultural Experiment Station, New Haven, Connecticut under Dr. Jason C. White's supervision starting from July 21, 2014. Mr. Mukherjee's dissertation title is: "*Impact of zinc oxide nanoparticles on green pea plant & seed quality and effects on physiological traits of green peas, corn, and zucchini by silver nanoparticles"*, supervised by Prof. Jorge L. Gardea-Torresdey.

**ACHIEVEMENTS AND AWARDS**

- Phyto-Scholar 2013: Awarded by the International Phytotechnology Society (IPS) and the National Institute of Environmental Health Sciences (NIEHS).
- SNO Student Award 2013: Awarded by the Sustainable Nanotechnology Organization.
- Graduate School Dodson Research Grant, Fall 2013, UTEP.
- ACS Local Chapter Travel Grant: 2013, UTEP.
- Dodson Travel Grant, College of Science, UTEP, 2013.
- Travel Grant: College of Science, UTEP (two times).
- Qualified National Eligibility Test (NET, December, 2005) in Environmental Sciences; conducted by the UGC, Govt. of India. (Ranked among top 12 students in India).
- National Talent Search Examination, India (four times).

**PUBLICATIONS**

1. **Mukherjee, A.**, Peralta-Videa, J. R., Bandyopadhyay, S., Rico, C. M., Zhao, L., and Gardea-Torresdey, J. L. (2014) ZnO nanoparticles induce phytotoxicity and differential anti-oxidative stress response in green peas (*Pisum sativum* L.) cultivated in soil. *RSC Metallomics*, 6, 132-138.

2. **Mukherjee, A.**, Pokhrel, S., Bandyopadhyay, S., Mädler, L., Peralta-Videa, J.R., Gardea-Torresdey, J.L. (2014) A soil mediated phyto-toxicological study of iron doped zinc oxide nanoparticles (Fe@ZnO) in green peas (*Pisum sativum* L.), *Chemical Engineering Journal*, doi: http://dx.doi.org/10.1016/j.cej.2014.06.112
3. Rico. C.M., Lee, S.C., Rubenecia, R., **Mukherjee, A.**, Hong, J., Peralta-Videa, J. R., Gardea-Torresdey, J. L. Cerium oxide nanoparticles impact yield and modify nutritional quality of wheat (*Triticum aestivum* L.) *Journal of Cereal Science* (submitted).
4. Bandyopadhyay, S., Plascencia-Villa, G., **Mukherjee, A.**, Peralta-Videa, J.R., Rico, C., José-Yacamán, M., and Gardea-Torresdey, J.L. (2014) Comparative phytotoxicity of ZnO NPs, bulk ZnO, and ionic zinc onto the alfalfa-*Sinorhizobium meliloti* association in soil (Ready to submit).
5. **Mukherjee, A.**, Sun, Y., Morelius, E., Tamez, C., Bandyopadhyay, S., Niu, G., Peralta-Videa, J.R., White, J.C., and Gardea-Torresdey, J.L. A life cycle comparative phyto-toxicological study of bare ZnO, alumina doped ZnO ($Al_2O_3$@ZnO), and KH550 coated ZnO (KH550@ZnO) nanoparticles in green pea plant (*Pisum sativum* L.) and its seed quality (In preparation, 2014).
6. **Mukherjee, A.**, De La Torre-Roche, R., Torselli, M., Hawthorne, J., Tamez, C., Morelius, E., Gardea-Torresdey, J. L., and White, J.C. Uptake and toxicity of coated and uncoated Ag NPs to soybean, zucchini, green peas, and corn (In preparation, 2014).
7. Bandyopadhyay, S., **Mukherjee, A.**, Rico, C., Peralta-Videa, J. R, and Gardea-Torresdey J.L. Differential toxicological effects of $CeO_2$ and ZnO nanoparticles on alfalfa's (*Medicago sativa*) secondary metabolites using advanced spectroscopic techniques (In preparation, 2014).

## BOOK CHAPTER

- **Mukherjee, A.**, Peralta-Videa, J.R., Gardea-Torresdey, J., White, J.C. (2014). Effects and uptake of nanoparticles on plants. In: "Biophysico-chemical Processes in Environmental Systems- Engineered nanoparticles and the environment: Biophysicochemical processes and biotoxicity." International Union of Pure and Applied Chemistry (IUPAC), John Wiley and Sons, Hoboken, NY, USA.

## POSTERS AND PRESENTATIONS

1. Mukherjee, A. et al. Effects of bare and coated ZnO nanoparticles on green pea plants (*Pisum sativum* L.) cultivated in soil, SNO conference, Santa Barbara, CA, November 2013.
2. Mukherjee, A. et al. Comparative phyto-toxicological effects of bare and silane coupling agent (KH550) coated ZnO NPs on green pea plants (*Pisum sativum* L.) grown in soil, 10th International Phytotechnologies Conference, Syracuse, NY, October 2013.
3. Mukherjee, A. et al. Toxicity of bare ZnO NPs on green pea plants (*Pisum sativum* L.) grown in soil, 246th ACS National Meeting, Indianapolis, September 2013.
4. Mukherjee, A. et al. One pot, room temperature synthesis of ruthenium nano-cubes, ACS National Meeting, 2010.
5. Mukherjee, A. et al. Synthesis of different size ruthenium nanoparticles, Chemistry Annual Research Symposium 2010, Department of Chemistry, Western Michigan University.
6. Invited speaker in the International workshop on Chemistry and Chemical Engineering, WMU, 2009.
7. Demo (on Chromatography) presented to the high school students in WMU Chemistry Day, 2010.

## ACTIVITIES

- Board member of the President's Advisory Committee on Sustainability, UTEP.
- Chairman, UTEP Green Fund Advisory Committee. (http://sa.utep.edu/greenfund/)
- Reviewer of the American Chemical Society's (ACS) peer reviewed journal.
- Environmental awareness program for local school students.
- Volunteering science outreach programs for Transmountain Early College High School students.

## DIPLOMA & CERTIFICATION

- UTEP College Teaching Certification, UTEP (2011).
- Diploma in Information Technology, NIIT, India (2001).

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- University of California-Center for Environmental Implication of Nanotechnology (UC-CEIN).

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