Zinc Sulphide Nanoparticle as an alternative source of Zinc Micronutrient for Crop Growth and Yield: Application and Evaluation

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DEDICATED TO MY PARENTS

DECLARATION

I hereby declare that this thesis entitled **"Zinc Sulphide Nanoparticle as an alternative source of Zinc Micronutrient for Crop Growth and Yield: Application and Evaluation"** represents my original research work and has not been submitted elsewhere for any degree, diploma or other qualifications. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

The work was carried out by me under the guidance of Dr. Prasanta Kumar Biswas at the Jadavpur University, Kolkata, India and Dr. Abhishek Mukherjee at the Indian Statistical Institute, Giridih, India.

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CERTIFICATE

This is to certify that the thesis entitled **"Zinc Sulphide Nanoparticle as an alternative source of Zinc Micronutrient for Crop Growth and Yield: Application and Evaluation"** submitted by **Mala Thapa (Reg. No.: 101/18/lifeSc./25)**, in partial fulfilment for the award of Ph.D. degree of Jadavpur University, comprises the work done by the student under our guidance at the Jadavpur University, Kolkata and the Indian Statistical Institute, Giridih, Jharkhand. It is hereby declared that the work is original and has not been submitted in full or in part for any degree, diploma or fellowship of any other University or Institution.

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List of Abbreviations

NPs : Nanoparticles

ENPs : Engineered Nanoparticles

nm : Nanometer

MPs : Micro-particles

$nTiO_2$: Nano Titanium Oxide

nAg : Nano Silver

$nZnO_2$: Nano Zinc Oxide

$nCeO_2$: Nano Cerium Oxide

nCu : Nano Copper

nCuO : Nano Copper Oxide

nAl : Nano Aluminium

nNi : Nano Nickel

nFe : Nano Iron

nZnS : Nano Zinc Sulphide

SiO_2 : Silicon Oxide

ZnO : Zinc Oxide

$Ni(OH)_2$: Nickel hydroxide

Cu : Copper

CeO_2 : Cerium Oxide

TiO_2 : Titanium Oxide

Fe_3O_4 : Iron Oxide

Au : Gold

List of Abbreviations

Ag	: Silver
Fe	: Iron
Zn	: Zinc
Mn	: Manganese
CdSe	: Cadmium selenide
ZnS	: Zinc Sulphide
ROS	: Reactive Oxygen Species
MWCNTs	: Multi walled Carbon Nano tubes
C70	: Fullerene carbon nanoparticle
Mn NPs	: Manganese nanoparticles
$MnSO_4$	: Manganese Sulphate
EPAs	: Environmental protection agencies
ZnSe	: Zinc Selenide
Mn^{+2}	: Manganese ion
QDs	: Quantum dots
h	: Hour
C	: Centigrade
M	: Molar
ppm	: Parts per million
DPPH	: 2,2-diphenyl-1-picryl-hydrazyl-hydrate

List of Abbreviations

min	: Minutes
CO_2	: Carbon di-oxide
S	: Sulphur
OH	: Hydroxy group
m	: Minute
s	: Second
rpm	: Revolutions per minute
KCL	: Potassium chloride

CHAPTER 1

Introduction to Nanotechnology, Engineered Nanoparticles in Agriculture and Zinc Sulphide Nanoparticles

1.1. Introduction to Nanotechnology

Today nanotechnology has emerged as a rapidly advancing field that showed promise in solving current economic problems through innovative products and their applications. The fabulous nanotechnology has revolutionized the whole of science and technology. It is an interdisciplinary subject which infuses scientific branches from biology, chemistry, physics and engineering. It deals with creation of novel nanoparticles (NPs) or products at the atomic, molecular or macromolecular level using a nanometer (nm) scale of approximately 1 to 100 nm at least at one dimension. At nanometer (nm) size matters exhibit novel and superior properties due to their smaller size and greater surface area. Therefore, the nanotechnology deals with the properties of a material that changes strikingly when the size reduces [1].

Engineered nanoparticles (ENPs) can be synthesized using "bottom-up" method (self-assembly) that create NPs from atoms and molecules or "top-down" method (milling) that create NPs from bulk materials using physical, chemical and biological mode of synthesis. They are synthesized to have greater functions which are not present in its bulk form of the same material [2]. ENPs may maintain the crucial characteristics of their bulk counterparts; so, considering bulk material during the testing is important. For instance, many studies have reported the harmful impact of heavy metals to the plants but silicon was found to be useful for plant growth [3-5]. ENPs can be synthesized

in various shapes and sizes according to need with suitable surface molecules which make them distinct from naturally occurring NPs or natural NPs (forms of minerals, clays, products of microbes etc) indicated in **Figure1** [6, 7]. Moreover, available data suggest that the ENPs are biologically more active than micro-particles (MPs) due to their size difference [8]. Hence, the unprecedented capabilities to control and characterize materials have increased its applications in various sectors like medicine, agriculture, food, pharmaceutical industries etc., which have also increased its emission into the environment [9, 10]. From the last decades, ENPs have influenced economy due to its potential application in different fields like cosmetic, textile, drug delivery, cancer treatment, biomedicines, waste water management, electronics, paints, sensors etc., [11, 12]. Various companies are synthesizing novel ENPs to improve products. For examples, ENP infused batteries showed improved functions such as faster charging, higher efficiency and higher power and are light-weight and safe [13]. Above 1800 nanomaterial products are present in the market [14]. In food industries, nanotechnology has been implicated significantly such as in consumer products; including packaging, food quality and freshness etc., [13]. However, compared to other sectors like drug delivery and pharmaceuticals, applications of nanotechnology to the agriculture and food sector are relatively recent [15]. Moreover, a few of the many ENPs are used in the industries on a very large scale. For examples, nano titanium oxide ($nTiO_2$), nano silver (nAg), nano zinc oxide (nZnO), nano cerium oxide ($nCeO_2$), nano

copper (nCu), nano copper oxide (nCuO), nano aluminium (nAl), nano nickel (nNi), and nano iron (nFe) are most commonly used ENPs in industries [16]. Also, semiconductor NPs such as nano zinc sulphide (nZnS) is extensively used in paints and rubber industries [17]. Therefore, release of these ENPs to the environment can be expected henceforth, more ENP-plant interaction studies are needed. Among different ENPs, metal or metal oxides ENPs have been studied on different plants. On the other hand nZnS is least studied NPs on biological system. Its effects on biological system are still unknown. Hence, the wide application of ENPs and the potential accumulation in the environment demands long term study on the effects of different kinds of ENPs on biological system.

Environmental conditions can affect ENPs characteristics by changing their aggregation state, oxidation, precipitation of secondary phases etc., [18]. Their reactivity with bio-molecules influence by several factors such as size, shape, material composition, surface functional groups, stability etc. [19, 20]. Also, surface coating of ENPs influences its responses compared to the bare ENPs [21]. Again, the physiochemical characteristics and chemical bonding in different medium influence their firmness. Hence, ENPs may act differently in different environment because their availability and reactivity affected by different medium [18].

To date, toxicity concerns of ENPs have raised and attracted several researchers. Various strategies are developed to evaluate the release of ENPs into the environment and their toxicity aspects [22, 23]. According to Europe consumption report, release of ENPs has been distributed significantly [23]. ENPs can enter to the different parts of the ecosystem such as fresh water, air, soil, etc., via direct application (as fertilizer, pesticides, for remediation of contaminated soils) or accidental release from the industries [24-26]. Moreover, these ENPs finally descend into the soil, may be harmful to the soil microbiota and to the plants as well, indicated in **Figure1** [25]. After uptake by plants, ENPs can be transported to different parts and may cause damage [27]. However, detail toxicity studies dealing with different ENPs are required to get a comprehensive picture. While, research on ENPs toxicity is increasing, many scientists reported toxicity of ENPs on human cell lines, bacteria etc., [28, 29] very few studies have been conducted with ecological terrestrial species such as plants [30]. Also, studies concerning the effect of long-term exposure of ENPs on plants are insufficient. Thus, there is a knowledge gap on long term exposure of ENPs on plant production, accumulation in edible parts, and nutritional qualities. Hence, there is a need to develop a deeper understanding of nature, fate and behaviour of ENPs in the plant systems.

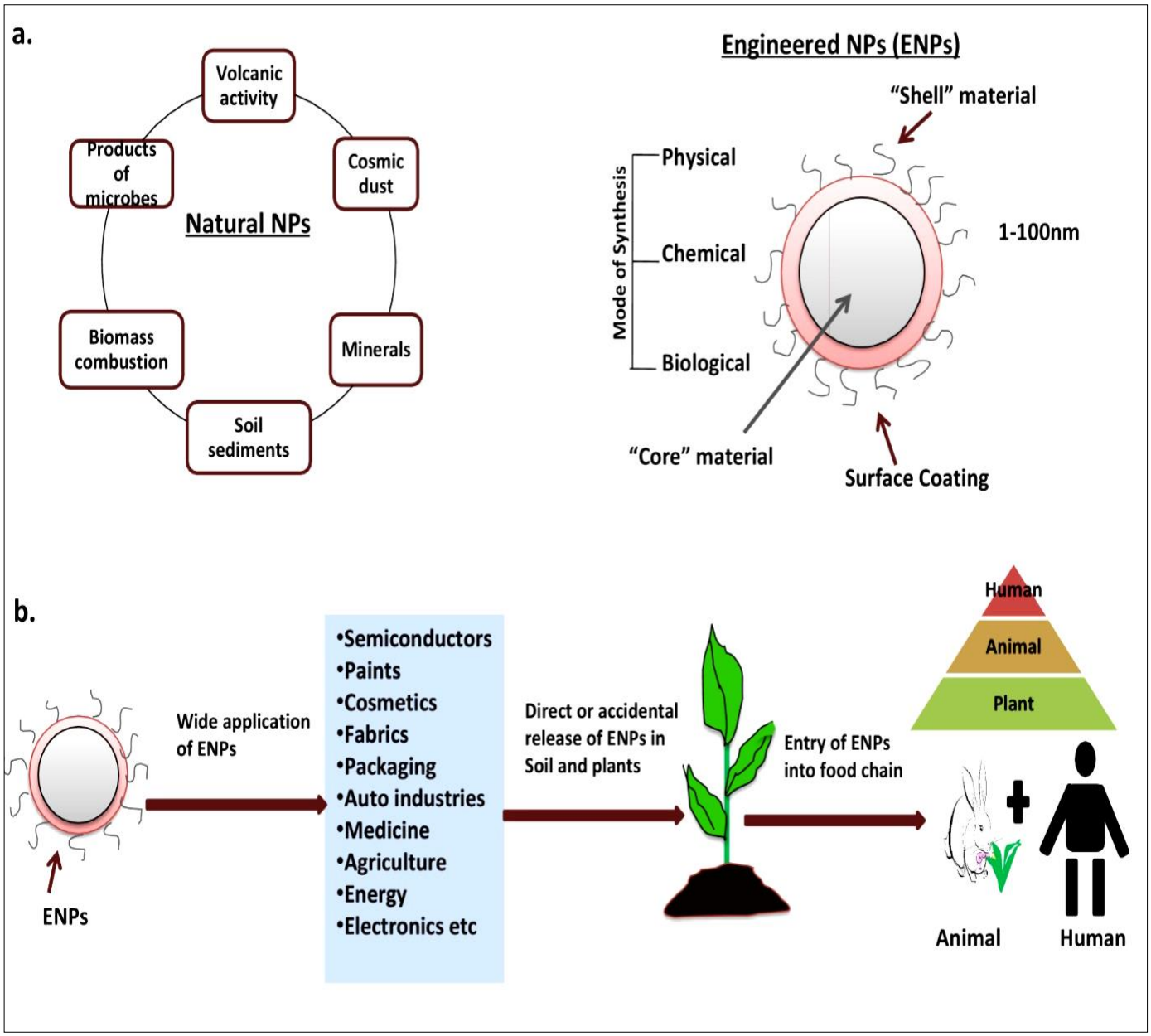

Figure1. (a) Natural and engineered nanopartcles (ENPs) and (b) Different applications of ENPs and its release to the environment.

1.2. Engineered Nanoparticles in Agriculture

Environment conditions influence ion concentrations in crop plants [31, 32]. Plants can absorb essential or nonessential elements under different environmental conditions, which beyond certain concentrations may cause toxicity to them [33]. Today, wide applications (either direct or indirect) of ENPs have received great concern toward their potential impacts on crops. The direct use of ENPs for targeted nutrient delivery to plants began in 1990 [34]. However, till now fate and activity of ENPs inside the plants are not cleared due to lack of well established techniques/protocols [35]. In addition, different elements with unknown functions are found to be stored in the plant tissues that can be translocated from plants to others [36, 37]. For example, plant that contains selenium can be used as a source of selenium to the deficient organisms [38]. Moreover, plants are interface between humans and their environment. As the plants are crucial in the transportation of ENPs in the food chain [39], understanding the effects of exposure to ENPs in plants is therefore, crucial. Recent studies have also demonstrated transfer of ENPs into aquatic food chain [40-44]. The different compositing elements or characteristics of ENPs may affect their activity, uptake, transportation and responses, for example, Barrios et al. (2016) has shown different response of plants to the same ENPs capping with different elements [21]. However, scientific

investigation on uptake, accumulation and effect of ENPs in edible plants are still scarce. Few reports have so far demonstrated the effects of ENPs on plant agronomic traits like biomass production, enzyme activity, photosynthetic processes, oxidative stress, and DNA expressions. The effects of different ENPs such as silicon oxide (SiO_2), zinc oxide (ZnO), nickel hydroxide ($Ni(OH)_2$), copper (Cu), cerium oxide (CeO_2), titanium dioxide (TiO_2), iron oxide (Fe_3O_4), gold (Au), silver (Ag), iron (Fe), and CdSe/ZnS quantum dot (QD) nanoparticles (NPs) on several crop plants like wheat, mungbean, alfalfa, tomato, corn, and cucumber [45] have studied, revealed not only negative but also positive or inconsequential results.

ENPs can block pores and inhibit the apoplastic movement. Due to the blockage caused by ENPs, hindrance in nutrients uptake [46] occur. ENPs toxicity include reduction in photosynthetic processes, Reactive Oxygen Species (ROS) generation, lipid peroxidation, oxidative stress, DNA and protein damage [47]. Moreover, ROS generation mechanism is primarily studied for ENPs toxicity [48, 49]. Also, ENPs can cause toxicity at high concentrations hampering crops productivity by changing their anatomical, physiological, biochemical and genetic aspects [50, 51]. After accumulation inside the plant, ENPs can degrade the crop's quality by reducing the seed germination percentage, fresh and dry weights and length of roots and shoots, changing the physiochemical pathways like respiration, transpiration, photosynthesis etc., changes genes level and

finally apoptosis. Once the ENPs enter into the plant, it can produce huge amount of ROS. These ENPs inside the plant cell can interact with different cellular organs and may hinder electron transport system, promote protein modification and induce oxidative burst [52, 53], as mentioned above. Despite of their destructive activity, ROS are required for cellular signal mechanism including plant growth and tolerance to environmental stresses [54]. There is a thin line of equilibrium presence between ROS generation and scavenging, based on which destructive or signalling roles of ROS occur. Plants naturally have some defense mechanisms to overcome stress conditions. Plants have different enzymes like superoxide dismutase (SOD), catalase (CAT) ascorbate peroxidise (APX) and guaiacol peroxidise (GPX) and antioxidants like flavonoids, carotenoids, tocopherols, and phenolics to fight agains oxidative stresses [50, 54]. When, ROS level increases in the plant cell the defense system activates. In this line, various studies have demonstrated the plant's increased enzymatic and antioxidant activity with oxidative stress due to exposure of ENPs, which confirmed the activation of plant defense system as a response to ENPs toxicity [53, 55, 56], while, the understanding of signalling pathways between ENPs and ROS is less explored. Also, the recent studies have shown significant function of photoreceptors in plant stress response signalling. Again, various hormones also play a vital role in stress signalling by up and down regulation [57]. But beyond a threshold level, break down of internal defense mechanism occur which eventually lead into apoptosis, elaborated in **Figure2**

[58]. In this line, few studies have shown changes in genes regulation due to stress e.g., drought, but till now effect of ENPs on gene regulation is not clear. Therefore, excess amount of ENPs produce ROS inside the plant cell, may induce oxidative stress, however, there is still a knowledge gap on other mechanisms which may play vital role in plant-ENPs interactions. Hence, much work is required in this field.

Moreover, for toxicity studies high concentrations of ENPs were used [59, 60], that may led to misleading conclusions on ecotoxicity study of ENPs which may inhibit the potential beneficial roles of ENPs. Most recent reports have evidenced positive as well as negative effects of ENPs in plants. For example, López et al. (2017), showed that nZnO at 400 mg kg^{-1} reduced seed germination and root length by 40 and 47% in maize (*Zea mays*) [61]. Lin and Xing (2007) showed that nZnO at 2000 mg L^{-1} concentration reduced seed germination and root elongation of ryegrass [62]. Lee et al. (2013) reported retardation of buckwheat root growth by nCuO and nZnO at 2000–4000 ppm [63]. Zhang et al. (2015) reported growth retardation of a wetland plant by nZnO treatments [64]. Stampoulis et al. (2009) reported that multi walled carbon nano tubes (MWCNT) at 1000 mg L^{-1} concentration reduced biomass of *Cucurbita pepo* by 60% [65]. Also, Lin et al. (2009) demonstrated that in *Oryza sativa* flowering was delayed by a month due to exposure of MWCNTs and C70 NPs [66].

Conversely, Awasthi et al. (2017), reported that nZnO treatment at 50 mg L^{-1} improved seed germination and plant biomass in wheat (*Triticum aestivum*) [67]. Mahajan et al. (2011) reported that nZnO at 1 to 20 ppm concentration promoted mungbean and chickpea growth [68]. Pradhan et al. (2013) demonstrated manganese nanoparticles (Mn NPs) were better than the commercially available $MnSO_4$ salt at 0.05 ppm concentration [69]. Siddiqui and Al-mutairi, (2014) reported that $nSiO_2$ (12nm) at 8 g L^{-1} enhanced seed germination of *Lycopersicon esculentum* [70]. Suriyaprabha et al. (2012) reported that SiO_2 NPs (20-40nm) treatment increase germination rate, root growth, dry weight and nutrient alleviation in *Zea mays* [71]. The majority of the ENPs showed biphasic dose response with low dose growth stimulation and a high dose inhibition in plants. Therefore, smartly designed ENPs can be used in agriculture and food sectors as growth stimulators, nanopesticides, nanofertilizers etc., [20, 72]. Thus, the selection of doses of NPs play vital role in NPs plant interaction study.

Additionally, all over the world, environmental protection authorities control the presence of toxic metals in the ecosystem. Therefore, presence of ENPs in the environment higher than the threshold level triggers remediation process by the authorities. Therefore, to claim an element toxic toxicity study at low concentration is needed. Hence, toxicological studies of ENPs at low doses are significant [73]. Moreover, each organisms including plant require only trace

amount of nutrient for metabolic process. Therefore, ENPs at low concentrations could be used as micronutrient to enhance their growth. ENPs have advantages over their bulk counterparts due to their superior activity, higher stability, and higher effectiveness in low doses [74]. Therefore, evaluating the beneficial roles of ENPs on biological systems is very important. Moreover, maximum amount of applied fertilizers never uptake by the target organism and eventually wasted [75], beside damaging many non targeted organisms. Therefore, targeted delivery of fertilizers would be a great idea that will reduce the wastage and make the agriculture more sustainable. Thus, an smart or advance technology is required to make environment friendly and cost effective fertilizers that can be easily uptake by plants [76].

ENPs mediated crop management has of late found potential applications [77]. Many papers have discussed the slow release of micronutrients from ENPs to plants. This approach has already been successfully implemented to deliver drugs, but there is few works has been done with plants. For instance, a polyphosphate micronutrient fertilizer was developed that can slowly release the micronutrient (Zn, Fe, Mn, and Cu) in acidic environment. This fertilizer can increase the rice productivity by 17%, in comparison with the commercial salts [78]. ENPs with slow-release capabilities could potentially lower the amount of micronutrient use and increase the bioavailability to plants. Also, it can minimise the waste due to running off the soil. However, studies related to

ENP-plant interactions and uptake-translocation in plants are lacking. Therefore, the future use of nanotechnology in agriculture require more studies related with plant-ENPs interactions, beside prioritizing certain ENPs [79].

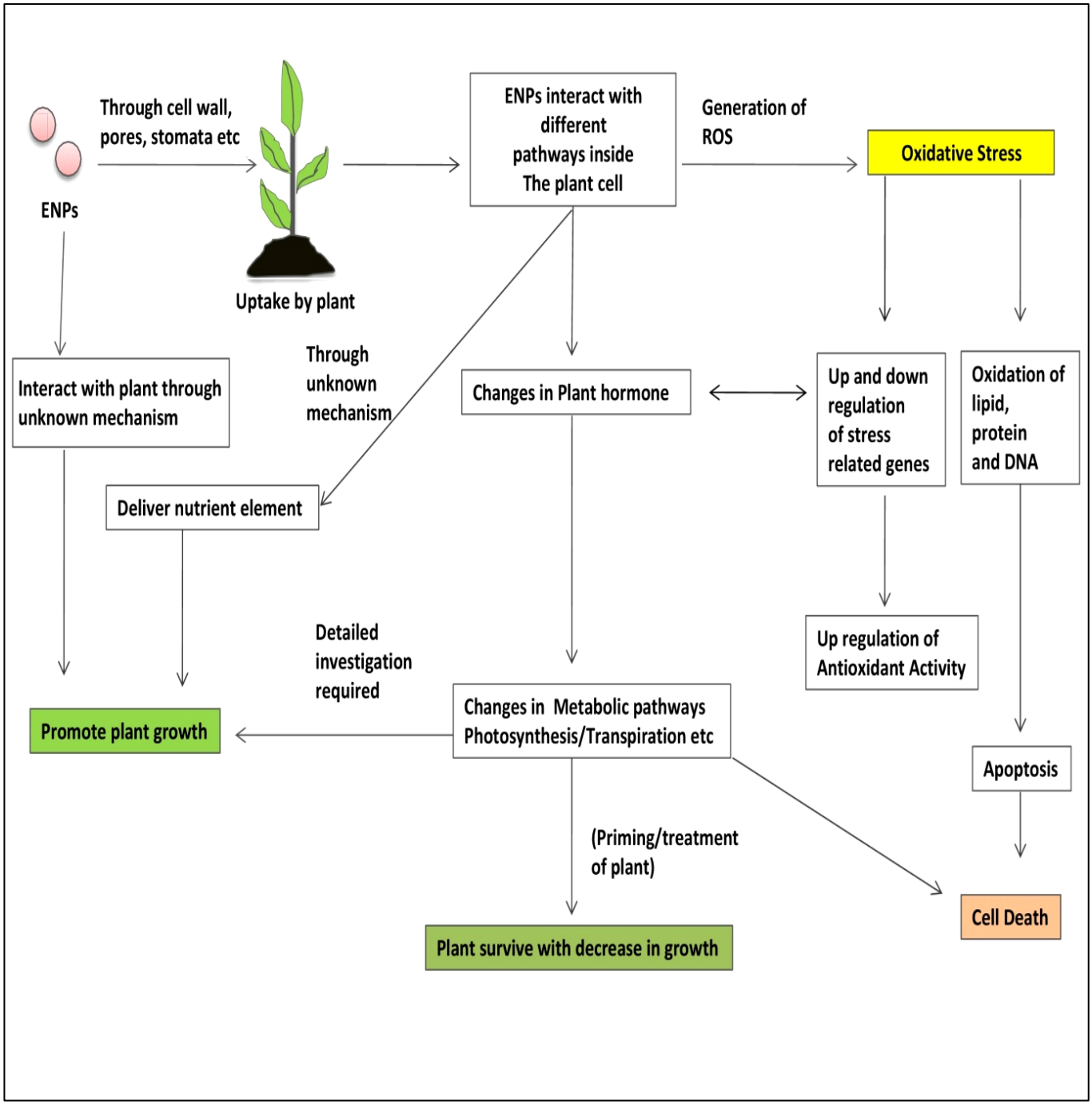

Figure2. Schematic representation of general mechanisms of ENPs and plant interactions.

1.3. Uptake and Translocation of Engineered Nanoparticles

The current studies on ENPs-plants interaction are more oriented to the effect of the NPs on plant's metabolic pathways such as impact of ENPs on germination, growth, physiological parameters etc. The uptake of ENPs by plants is a new field of study with embedded challenges. The key problem to study ENP-plant interaction is to detect the internalized ENPs within plant tissues with their atypical characteristics. Recent studies revealed that multiple factors including species, size, shape, surface composition etc., influence uptake and accumulation of ENPs [47]. When plant comes in contact with ENPs, they can adhere to the cell wall and penetrate through the epidermis (**Figure 3**). A multiple events occur following entering into the vascular system (xylem), and then transfer to stele, to be translocation to different parts. In this process, plant cell wall acts as a gateway for ENP entry. Cell wall facilitates the entry of tiny particles while restrict the large particles, hence smaller NPs can go easily through plant cell wall. Thus, the uptake of ENPs by plant cell is size specific. The size exclusion limit for the plant cell wall is between 5 - 20 nm [46]. While few ENPs can form bigger pores which further accelerate the entry of large size ENPs [80, 81]. Also, ENPs may enters the cell through endocytosis process [82], and inside the cells it may follow apoplastic or symplastic pathways throughout the plant system. Wong et al. (2016) in his recent study, proposed a

model that can explain the mechanism of lipid modification during ENPs entry inside the plant cells [83]. Again, ENPs can bind with membrane protein transporters to be entering into the plants. Also, ENPs may transport from one cell to the other within plants through plasmodesmata. Moreover, xylem plays key role in the transportation of ENPs [84]. For instance, Wang et al. (2012) reported uptake and translocation of nCuO though xylem-phloem transport system in *Zea mays* L. [85]. ENPs entries into the plant cell follow an active transport system which include other cellular signalling pathways also
[86]. Also, some studies have reported the entry of ENPs into plant cells via endocytic pathways [87]. For instance, Onelli et al. (2008) reported that Au NPs entry into *Nicotiana tabaccum* was clathrin independent as well as clathrin dependent [88]. Again, ENPs uptake in seed occur through diffusion method in intercellular spaces of parenchyma [89, 90]. In the seed, aquaporins transporter also facilitate the entry of ENPs [91, 92]. For instance, CNT treatment induced up regulation of aquaporin proteins in *Lycopersicon esculentum* [92, 93].
In case of leaf uptake, ENPs enter into the plant cells via cuticle hair and stoma [94]. Again, when ENPs expose in plant leaves, they gather in the stomata following translocation to different parts via phloem. For instance, Eichert and Goldbach (2008) demonstrated hydrophilic particles (≥40 nm) transfer through stomata pores [95]. Infect different plant transport system regulated by multiple parameters, such as size of the pores, hydraulic conductivity etc.

In the recent years, much works has been done to understand the uptake and translocation mechanism of ENPs, which can be used to detect the ENPs translocation kinetics. From literature review, it is clear that ENPs can transport inside the plant cells through (1) aquaporins, (2) carrier proteins, (3) ion channels or endocytosis, (4) new pores and (5) by binding to organic chemicals in the environment. However, some questions are yet to be answered, such as (i) how do these ENPs penetrate the cell wall, (the specific mechanism behind)? (ii) whether they will transport via symplastic or apoplastic pathways? (iii) how do they traverse the endodermis casparian strip? (iv) why only some plant readily uptake several ENPs [47]. Till date, the published data about ENP uptake by plants is still not conclusive. Therefore, detailed research is needed to address the uptake, accumulation and translocation mechanism of ENPs inside plants.

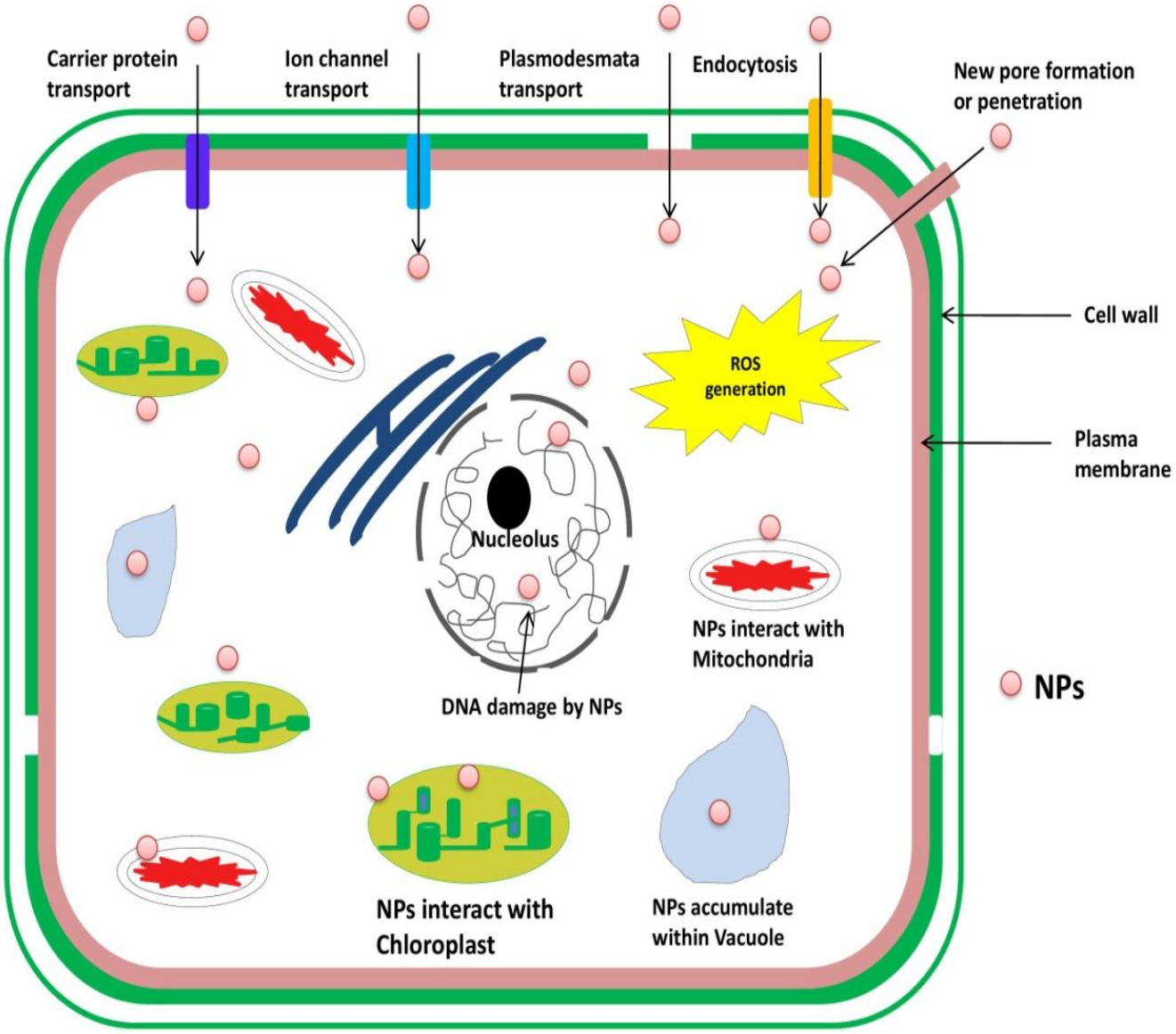

Figure3. Probable modes of cellular uptake and interaction of ENPs with different organs inside plant cell.

1.4. Zinc Sulphide Nanoparticles

ZnS is a natural salt which is the main source of zinc in nature. It is a tetrahedral polymorphous material naturally exists in two common crystalline forms i.e., zinc blende (sphalerite) and wurtzite forms. Zinc blende (sphalerite) is a cubic crystal form which is more stable and predominates in nature. Wurtzite has a hexagonal crystal structure, although it is scarce in nature, can be produced synthetically. The transformation from the zinc blende to the wurtzite form can be done by heating at around 1020° C. Naturally, ZnS appear black in colour due to the presence of iron in it but the purify form of ZnS are white or pale yellow or gray in colour. It is usually produce from waste materials. Again, it is the first semiconductor that can be used for diverse applications. It is commonly use as white pigment for paint, plastic and rubber. Also, it is use as phosphor in several applications, such as X-ray, cathode ray tubes and glow in dark products. It is a direct band gap II-VI semiconductor which contains wide band gap value (≈3.54 eV for zinc blend and 3.91 eV for wurtzite) than ZnO (3.4 eV). Because of its large band gap size it is considered as a perfect choice for visible and UV light based devices. It is an efficient photocatalyst also, that can be used to degrade the harmful products like hair dye etc., through enhancing the light absorption capacity. It is more stable and much better than its alternative chalcogenides (such as ZnSe), hence is used as a favourable host material. All these characteristics are maintained when the material is featured to nano

dimensions. Moreover, in comparison to bulk form, nano-ZnS (nZnS) exhibit atypical physiochemical characteristics including large surface to volume ratio, the quantum size effect, more optical absorption, chemical reactions and heat resistance capacity, catalysis, and the low melting point. Therefore nZnS are interesting material for versatile applications.

Zinc sulphide nanoparticle (nZnS) is widely used nanomaterial among the different semiconductors [96]. It has promising nanoscale properties then other semiconductors. For instance, Kripal et al. (2010) demonstrated the synthesis of Mn^{+2} doped ZnS by co-precipitation method and showed its photoluminescent and photoconductivity characteristics [97]. Likewise, Kanemeto et al. demonstrated the photo-physical and photocatalytic properties of nZnS [98]. nZnS are nontoxic and more photostable in nature than other semiconductors [99]. It has catalytic properties that can remove toxic substances from water or environment. For instance, nZnS used in waste water treatments as a photocatalytic agent to degrade dyes, [100]. Again, due to its larger band gap value it can be used in both biomedical and optoelectronic applications [101]. Its atomic structure and chemical properties are comparable to more popular and widely known ZnO. However, the nanostructures of ZnS have not been investigated in much detail relative to ZnO nanostructures. In the recent years, nZnS have been used as biosensors. They are used as real time sensors for biological species. For instance, enzyme conjugated CdSe/ZnS core-shell QDs is used to detect glucose. This QD system possesses superior design, high

flexibility, low cost and good sensitivity [102]. Therefore, extensive industrial applications of nZnS aggravate its chances of release to the soil or environment. Therefore, more studies are warranted to shed light on impact of nZnS on plant systems as plants are the primary producers.

1.5. Conclusion and Perspectives in the Present Study

From the perusal of the literature, it is understood that the plant-ENPs interactions is a composite phenomenon which depends on several properties of ENPs as well as plant species. The key factors which play vital role during plant-ENPs interactions are size, shape, surface functional group, structure, concentration, treatment methodology, plant species, soil type, illumination intensity, time and route of exposure. Again, ENPs can cause toxicity at high concentration while at low concentration they can be used as growth regulators. There are many studies showed that at low concentrations, ENPs significantly increased the plant growth, biomass and enhanced the nutritional qualities which indicated their beneficial roles in agriculture as nanofertilizers. ENPs can positively affect photosynthetic yield, fruit and flower yields. Therefore, nanomaterials or nanotechnology would be a great approach in future agriculture. But, before doing any experiments with the plants, both positive and negative responses of ENPs should be considered.

Research on understanding the mechanism of toxicity, uptake, translocation and biotransformation of ENPs on edible crops are very recent field of study. Limited papers are present on the mechanism of uptake and translocation of ENPs within plants. Therefore, the safe use of ENPs in agriculture needs a thorough study of their interaction with edible plants, at physiological,

biochemical and molecular level. Recently, researchers are giving efforts to understand the uptake and translocation mechanism but the associated modes of action are yet to be known. Meanwhile, there are very few studies present which showed the long-term effects of ENPs on plants. Thus, little is known about the effects of prolong exposure of ENPs on plant production, ENPs accumulation in edible/reproductive organs, and nutritional value of edible tissues. Moreover, different types of ENPs have been studied for better understanding the ENPs-plant interactions but less effort has been expended on impact of zinc sulphide nanoparticle (nZnS) on crop plants. In spite of extraordinary properties of nZnS, its role on plant system is not well understood, yet. The concise discussion and mechanisms through which plant reacts to nZnS is not clear yet. Also, it is noteworthy to see if nZnS could be used as nanofertilizer in future. Hence, the above information emphasise that detailed studies on the mechanism of uptake, translocation and effects of nZnS application in plant growth and yield would be necessary to understand the gamut of nanotechnoly in agriculture.

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CHAPTER 2

Zinc Sulphide Nanoparticle (nZnS): A novel Nano-Modulator for plant growth

2.1. Introduction

Engineered Nanoparticles (ENPs) have emerged as one of the most innovative and rapidly growing fields in industries, agriculture and medicine sectors. Because of their unique physicochemical and optical properties, ENPs are expected to be biologically more active than their bulkier counterparts. Recent reports indicated that more than thousands of commercial products use ENPs (Berube et al., 2010). As plants play vital role in the transportation of ENPs in the food chain through uptake and bioaccumulation (Rico et al., 2013), understanding the effects of exposure to ENPs in plants is therefore, crucial. However, scientific investigation on uptake, accumulation and effect of ENPs in edible plants are still scarce. Few reports have so far demonstrated the effects of ENPs on plant agronomic traits like biomass production, enzyme activity, photosynthetic processes, oxidative stress, and DNA expressions. Prior work with plants has evaluated the toxicity of silica (SiO_2), zinc oxide (ZnO), nickel hydroxide ($Ni(OH)_2$), copper (Cu), cerium oxide (CeO_2), titanium dioxide (TiO_2), iron oxide (Fe_3O_4), gold (Au), silver (Ag), iron (Fe), and CdSe/ZnS quantum dot (QD) nanoparticles (NPs) on *Arabidopsis thaliana*, ryegrass, mesquite, and select edible plant species including wheat, mungbean, alfalfa, tomato, corn, and cucumber (Slomberg and Schoenfisch, 2012). Most recent reports have evidenced both positive and negative effects of ENPs in plants. For instance, López et al. (2017), showed that nZnO at 400 mg kg^{-1} reduced seed

germination and root length by 40 and 47% in maize (*Zea mays*). Conversely, Awasthi et al. (2017), reported that nZnO treatment at 50 mg L^{-1} improved seed germination and plant biomass in wheat (*Triticum aestivum*). ENPs move via pore and the uptake and translocation of ENPs are shape, size and composition dependent (Zhang et al., 2015). Recent studies have been made to understand the uptake accumulation and translocation mechanism of ENPs. For instance, Wang et al. (2016), demonstrated the xylem and phloem mediated uptake, translocation and distribution of nCuO (20–40 nm) from root to shoot through the xylem and its reverse transport to root through the phloem in *Zea mays*. Overall, only a limited numbers of studies are available on uptake of ENPs by plant species that subsequently accumulate in the various cellular locations and alter different biochemical processes, to date (Pradhan et al., 2013; Ghafariyan et al., 2013; Lin and Xing, 2008; Yang et al., 2014). Therefore, detail studies to generate comprehensive pictures of ENPs interactions with edible plants at the physiological and biochemical levels are required for their safe use in agriculture.

Zinc sulphide nanoparticle (nZnS) is widely used nanomaterial among the different semiconductors (Fang et al., 2011). They are used in biological applications as tagging molecules (Jin et al., 2016), pharmaceuticals (Pathakoti et al., 2013), cosmetic and rubber industries (Bhattacharjee et al., 2013) and in paint (Womack et al., 2004). nZnS is nontoxic and more stable in nature than other semiconductors (Zaba et al., 2016). It has a wider band gap value than

large sized ZnS and nZnO. Because of these properties, nZnS can be used in both biomedical and optoelectronic applications (Suyana et al., 2014). Certain properties pertaining to nZnS, like it's small size with larger band gap, good biocompatibility and easy synthesis are unique and advantageous, making its commercial use economical (Huang et al., 2006). The extensive industrial applications of nZnS aggravate the possibility of its environmental dispersion and plant uptake. While some researchers have conducted mammalian and eco-toxicity studies of nZnS, like effects on retinal pigment epithelial cells (Karthikeyan et al., 2016) and on the crustacean *Daphnia* sp. (Lin and Xing, 2008), studies of its environmental fate including its effects on plant system are generally rare, with some exceptions. For example, applications of nZnS at 15 ppm concentration in *Brassica juncea* seedlings resulted in improved growth and antioxidant levels in the treated plants (Nayan et al., 2016). In another study, internalization and translocation of polymer coated CdSe/ZnS QDs was studied in *A. thaliana*. This study reported that polymer coated CdSe/ZnS QD was not internalized by *A. thaliana* (Navarro et al., 2012). Conversely, in Medicago sativa cells, bioaccumulation of CdSe/ZnS QD occurred specifically in the cytoplasm and the nucleus (Santos et al., 2010).

Cytotoxicity and genotoxicity studies are equally important for understanding toxicity of ENPs (Kumari et. al., 2011). The *Allium cepa* root chromosomal aberration assay is an established plant bioassay validated by the International Programme on Chemical Safety and the United Nations Environment

Programme as an efficient and standard test for the chemical screening and in situ monitoring for genotoxicity of environmental substances (Organization, W.H. 1985 and Grant, W.F. 1982). Literature review indicated a number of studies have used *Allium cepa* root chromosomal aberration assay to study toxicity of ENPs. For example, Kumari et al. 2011 reported the cytotoxicity and genotoxicity of nZnO in *Allium cepa*. Ghosh et al. 2015 reported cytotoxity and genotoxicity of MWCNT applications in *Allium cepa.* However, there are no reports present on cytotoxicity and genotoxicity effect of nZnS in plants till date.

Hence, the above information emphasise that detailed studies on the mechanism of uptake, translocation and effects of nZnS application in plants would be necessary to better understand its impact on plants. In view of the above information, the current study was conducted with the following objectives, (i) to study the role of nZnS application on plant growth and antioxidant defense, and (ii) to investigate the uptake and translocation of nZnS, and its effects on plant cell microstructure and (iii) toxicity study (if any).

2.2. Materials and Methods

2.2.1. Synthesis and physicochemical characterization of nZnS: nZnS was synthesized by a modified reflux method (Suyana et al., 2014; Fu et al., 2012). Briefly, 50 mL of 1M aqueous solution of zinc nitrate was refluxed under nitrogen atmosphere. 50 mL of 1M sodium sulphide solution was added in a drop by drop fashion to the above solution and allowed to stir for 6 h at 80 °C. After stirring, a white thick precipitate thus obtained was centrifuged at 10,000 rpm for 10 min and washed several times with an excess amount of Millipore-water and ethanol to remove any un-reacted species. Finally, the synthesized product was vacuum dried to obtain nZnS. Physicochemical characterizations of the synthesized particles were conducted by X-Ray Diffraction analysis (XRD, Cu Kα radiation, λ=1.5404 Å, X-PERT PRO diffractometer), Field Emission Scanning Electron Microscopy (ZEISS FE-SEMs), High Resolution Transmission Electron Microscopy (HR-TEM, JEOL JEM 2100 HR with EFLS), Fourier Transforms Infrared Spectra (FTIR, JASCO FTIR-6300), Photoluminescence (Perkin-Elmer LS55), Ultra-visible spectroscopy (UV-3600 series, Shimadzu), Dynamic light scattering (DLS) and Zeta potential (Malvern zetasizer).

2.2.2. Role of nZnS application on plant growth and antioxidant defense

2.2.2.1. Preparation of nZnS suspensions: Effects of nZnS on plants were studied using three different concentrations viz: 0.1, 0.5 and 1 mg L^{-1}. The choice of treatment concentrations was based on previous studies like Pradhan et al. (2013), Ghafariyan et al. (2013), and Mahajan et al. (2011), which reported the bioavailability of ENPs at low concentrations (few ppm). The nZnS suspensions were prepared by sonicating nZnS powder in Milliporewater at 25 °C for 1 h. Freshly, prepared suspensions were used each time for the treatments.

2.2.2.2. Plant material: Effects of nZnS application on plants were studied on mungbean seedlings (*Vigna radiata*). Mungbean seeds were purchased from Berhampur Pulse and Oil Research Centre, West Bengal, India and used as experimental material. Seeds were surface sterilized using 5% sodium hypochlorite solution (w/v) for 10 min followed by thorough and repeated washing by deionized water before experimental treatments.

2.2.2.3. Germination test and seedling growth condition: Surface sterilized seeds were imbibed with different experimental doses of nZnS (0, 0.1, 0.5 and 1 mg L^{-1}) and kept in dark for 4 h. After that, treated seeds (n=100) were kept in the Petridishes with filter paper moist with respective treatment solutions, for 24h in dark at 28 °C. After 24 h, number of seeds that has developed a primary root of at least 1mm long was counted and germination percentage was

calculated according to Singh et al. (2013). The experiment was conducted with five replications. For further experiments, germinated seeds were placed individually in square glass-plates (14×14 cm) lined by filter paper, moist with different treatment solutions (0, 0.1, 0.5 and 1 mg L^{-1}). The glass plates containing plantlets were then dipped into respective treatment doses (Saha et al., 2012) and monitored for next 10 days. The plants were watered regularly so that only the roots were submerged in the suspensions. The experiment was conducted in a completely randomized design (CRD); 5 replicates were used for each experiments and each replicate comprised a single glass plate containing 15 plantlets. Equal space was maintained between the plantlets to avoid competition among them. Plants were grown in a growth cabinet (GC-300, Lab companion) with 14 h photoperiod, 28 °C; night temperature of 20 °C and RH 40–60%, light intensity was 440 μmolm^{-2} s^{-1}.

2.2.2.4. Morphological parameters: The effects of nZnS on growth parameters were studied in terms of root-shoot length, fresh-dry weights of the plant, and rootlet numbers per plant. After 10 days, roots were rinsed with deionised water and plants were separated into roots and shoots, and their length, rootlet numbers and fresh weights were determined. Dry weights were recorded by drying the plants at 80 °C for 24 h.

2.2.2.5. Estimation of photosynthetic pigment content: Chlorophyll was extracted using buffered aqueous 80% acetone (pH 7.8) and was estimated by Arnon's formula (Arnon, 1949). Carotenoid content was estimated following Davis et al. (2003). Carotene and xanthophyll were measured by utilizing the values of absorbance at 425 nm and 450 nm respectively.

2.2.2.6. H_2O_2 generation, lipid peroxidation, proline content, electrolyte leakage and total protein content: The H_2O_2 content was analyzed according to Sinha (1972) protocol. Thiobarbituric acid (TBA) test was used to measure the Malondialdehyde content (MDA), the end product of lipid peroxidation (Hodges et al., 1999). Proline content was measured following Bates et al. (1973), method. Electrolyte leakage was determined according to Lutts et al. (1996), method. Total protein content was determined according to Lowry et al. (1951) and enzyme activity was expressed in terms of change in OD at 420 nm min^{-1} mg^{-1} protein.

2.2.2.7. Antioxidant defenses; phenol, flavonoids, total antioxidants and radical scavenging activity using DPPH: Phenol content was determined by the modified Folin-Ciocalteao method (Alhakmani et al., 2013). Flavonoids content was measured qualitatively following Ebrahimzadeh et al. (2010). Total antioxidant activities (TAA) were determined according to Prieto et al. (1999). DPPH radical scavenging activity was measured according to Blois (1958).

2.2.2.8. Enzyme assays: For the enzyme studies, plant material was extracted in 0.1M phosphate buffer (pH 7) at 4 °C. Then the extract was centrifuged for 25 min at 10,000 g, at temperature 4 °C. The supernatant was used for enzymatic assays; superoxide dismutase (Giannopolitis and Ries, 1977), catalase (Chance and Maehly, 1955), peroxidase (Thurman et al., 1972), glutathione reductase (Foyer and Halliwell, 1976) and ascorbate peroxidase (Nakano and Asada, 1981).

2.2.3. Uptake and translocation of nZnS, and its effects on plant cell microstructure

2.2.3.1. SEM and TEM study: For SEM, 10 days old fresh mungbean seedlings (control and treated) were thoroughly washed with deionized water. Roots and leaves were cut in thin transverse sections (T.S.) and fixed with 2% glutaraldehyde solution at 4 °C for 2 h, followed by postfixing the samples for 2 h with 1% osmium tetroxide solution. The samples were then dehydrated with graded ethanol. Then the roots and leaves sections were coated with platinum for 60 s (ca. 1 nm platinum layer) by using a Sputter Coater and then observed under SEM (JEOL JSM-7600F, with Energy Dispersive X-ray, EDX). For TEM, 1% paraformaldehyde (PF) along with 3% glutaraldehyde was used for

fixing following standard procedure (LIN, 2005). Then the samples were cut in T.S. using a microtome and observed under TEM, (Tecnai, G 20, FEI).

2.2.3.2. Zn release study by ICPMS: Zn release from nZnS was monitored by using Inductively Coupled Plasma Mass Spectroscopy (ICPMS, ELAN DRC-e, Perkin Elmer) at pH 7. For in vitro study, 100 mL of nZnS solution in Milli-Q water of treatment concentration (0.1, 0.5, and 1 mg L^{-1}) was allowed to stir for 24 h. After 24 h of stirring the supernatant was collected by centrifugation at 14,000 rpm for about 20 min. Finally the suspension were digested with ultrapure HNO_3 using standard methods (Pradhan et al., 2013), and then subjected to ICPMS using a Zn standard solution. For in vivo ICPMS measurement, after 10 days of treatment plants were harvested, thoroughly washed with tap water followed by rinsing with deionized water, air-dried, weighed, divided into two parts (roots and leaves), and sieved. Then the sieved plant materials were digested in a microwave accelerated reaction system using a mixture of plasma pure HNO_3 and H_2O_2 (1:4) and analyzed for Zn content.

2.2.4. Cytotoxicity and Genotoxicity Study

2.2.4.1. Effect of nZnS on *Allium cepa* root meristem: Equal sized *A. cepa* bulbs were purchased from local market. The bulbs were allowed to germinate in sterilized moist sand in dark room. The germinated bulbs (3 bulbs per exposure) with their roots (1-2cm) were then exposed to different

concentrations of nZnS (0, 0.1, 0.5, 1 mg L^{-1}) and maintained for 24hours in room temperature (28º ± 2º C), and 14 hrs photoperiod (light intensity 440 µmoles/m^2/s).

2.2.4.2. Cytotoxicity assay (Evans blue dye): Loss of cell viability was studied using the Evans blue staining method. *A. cepa* roots were stained with Evans blue (0.25% w/v) for 15 min and subsequently washed with DI H_2O for 30 min. Triton X-100 was used as positive control (+control). The roots were then macro photographed to estimate cell death. Additionally, 3 root tips (1 cm) were soaked in N,N-dimethyl formamide for 1 h at room temperature and the absorbance was measured at 600 nm (in UV-3600 series, Shimadzu).

2.2.4.3 Genotoxicity Assay (Chromosome aberration and micronucleus assays): The clastogenic/anneugenic effect of nZnS was evaluated on the basis of chromosome aberration test results in *A. cepa* root cells. Following exposure, slides were prepared from each of the roots according to the squash technique. Briefly, root meristems (10-15 in number), were chosen at random from five bulbs per exposure and were excised and fixed for 3 hrs in acetic acid/ethanol (1:3). The excised root tips were subsequently hydrolyzed and stained in a 9:1, 2% acetoorcein-HCl mixture. Slides were prepared from each of the root meristems and 1000 cells/root was scored. Maleic hydrazide (1µg ml^{-1}) was used as a positive control for the experiment.

2.2.4.4 DNA Laddering: DNA was isolated from control and treated *A. cepa* roots using a CTAB method DNA purity was determined by measuring the absorbance of the diluted DNA solution at 260 and 280 nm. The isolated DNA samples were resolved on 2.5% agarose gel in 1 × TAE (Tris-acetate-EDTA) buffer at 100 V, for 90 min at 4°C. The DNA was then stained with an aqueous EtBr solution, visualized and imaged under a UV trans-illuminator.

2.2.5. Statistical analysis

The data were expressed as mean ± standard deviation of five replicates. Statistical differences among treatments were determined using one-way analysis of variance (ANOVA) followed by Tukey's test at a significance level of 0.05.

2.3 Results and Discussions

2.3.1. Physicochemical characterization of nZnS: The XRD pattern confirmed crystalline structure of synthesized nZnS. Three characteristic peaks were obtained with 2θ=28.5°, 47.7° and 56.5°, indexing (111), (220), and (311) diffraction planes of nZnS (JCPDS card no. 05–0566); with cubic blend structure (**Fig. 1a**). Additional peaks of impurities were absent signifying nZnS phase purity. Meanwhile, the morphology of synthesized nZnS was detected using FESEM, showed its nearly spherical morphology, which were homogenous in shape, (**Fig. 1b**). HR-TEM image of synthesized nZnS justified the same spherical morphology. The sizes of the particles were ≤20 nm with an average diameter of 13.3 ± 0.3 nm, (**Fig. 1c**). The surface properties of nZnS were further analysed by the FTIR spectra. The surface of nZnS consisted of mainly three deep set (1127, 1010 and 662 cm^{-1}) in the transmittance spectra. The peaks at 1121 and 1001 cm^{-1} attributed to S-O stretching and the one that appeared at 660 cm^{-1} was assigned to the nZnS (Pathak et al., 2013). Also, the peaks cantered at 3400-3600 cm^{-1} (OH-stretching) because of some absorbed moisture and at 1628 cm^{-1} is due to the C=O stretching modes arising from the absorption of atmospheric CO_2 on the surface of the nanocrystals, (**Fig. 1d**). In addition, a small but negative zeta potential of −4.84 mV at 25 °C (pH 7) was observed which corroborated its surface functionality and stability. The UV absorption spectra revealed a characteristic hump shaped curve of nZnS, with a

strong absorption at 324 nm (band gap=3.82eV, **Fig. 1e**). The luminescence spectrum of nZnS upon excitation at 270 nm was presented in **Fig. 1f**. A luminescence peak at 445 nm was attributed to the defect state (S^{-2}) related to the emission from the nZnS host, while the peak at 530 nm was assigned to the S vacancy.

Prior to application the stability of nZnS was also checked with the aid of their hydrodynamic radius measurements which were found to be around 100 nm [**Fig. S1**]. The hydrodynamic radius justified that nZnS produced a stable dispersion, and the size of the dispersed particles remains well within the nano size range that could be used under hydroponic condition.

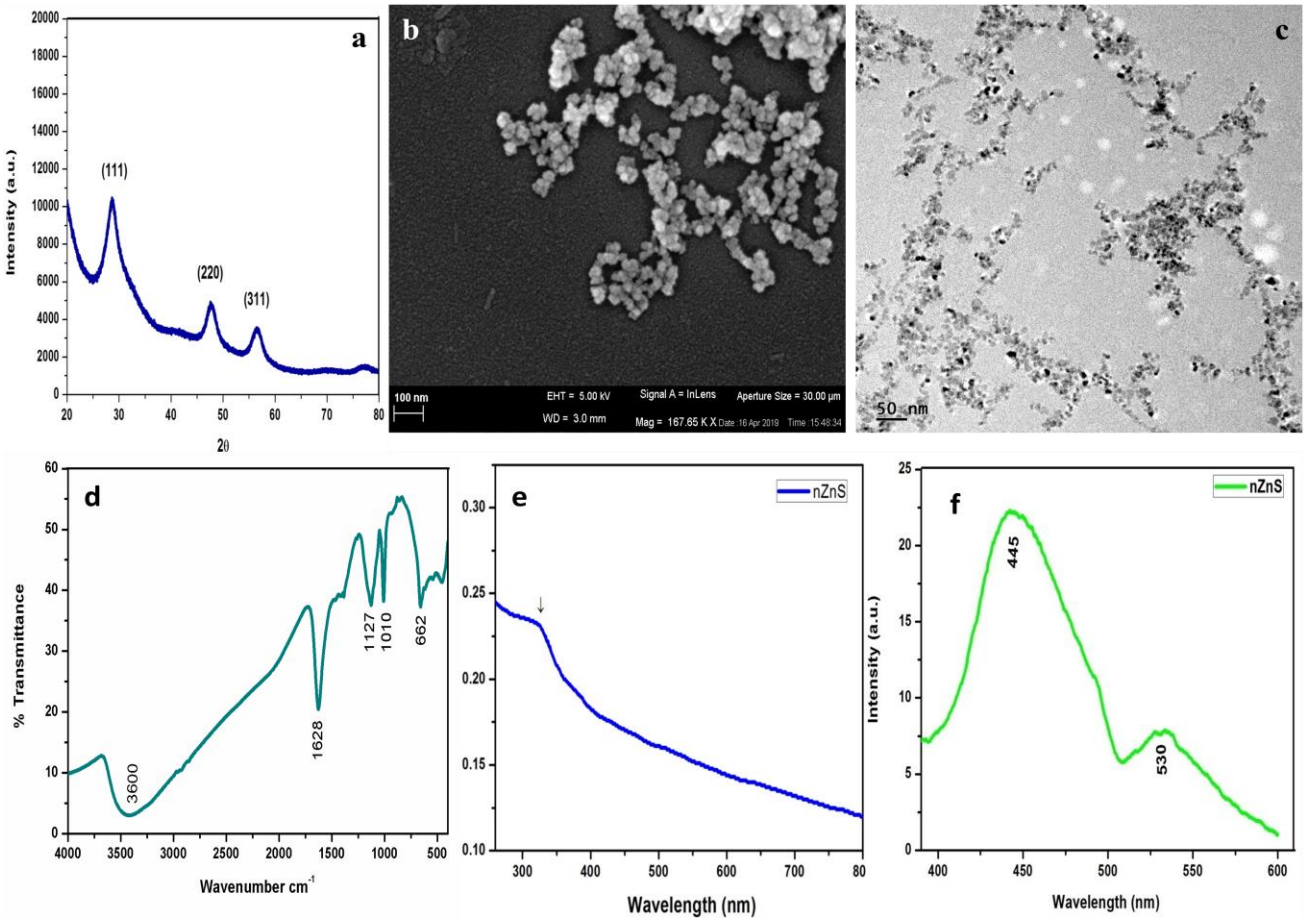


Figure1. (a) XRD pattern, (b) FE-SEM image, (c) FE-TEM image (d) FTIR (e) UV-spectroscopy and (f) PL spectra of synthesized nZnS.

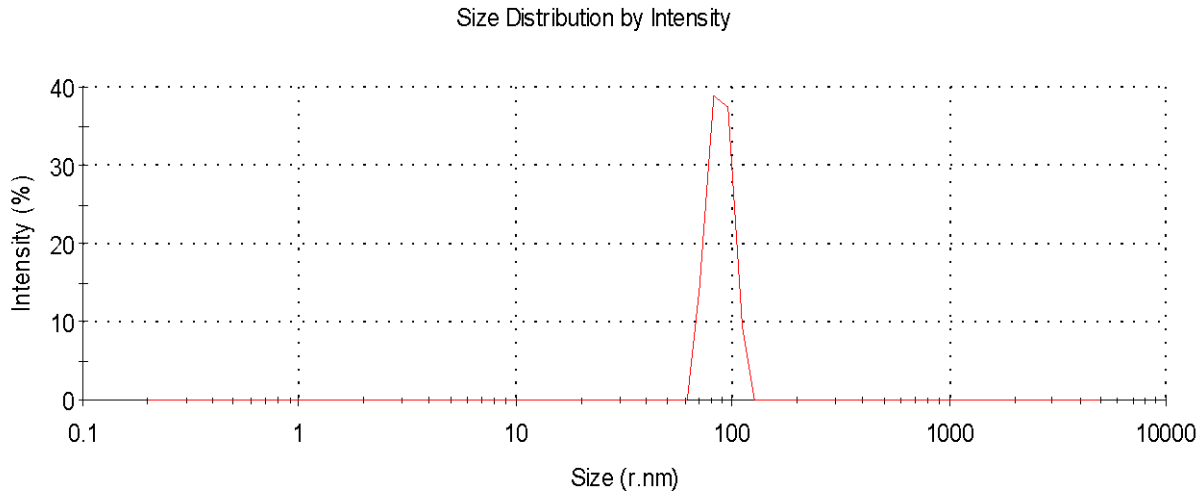


Figure S1. Hydrodynamic radius measurement of synthesized nZnS using DLS.

2.3.2. Role of nZnS application on plant growth and antioxidant defense

2.3.2.1. Seed germination and plant growth morphology: In the present study, seed germination and plant growth was positively altered by nZnS treatment. A significant (F=5.74 and p=0.007) increase in germination percentage of treated seeds over control was observed. The highest percentage of seed germination was 98%, occurred at 0.1 mg L^{-1} nZnS concentration. The treated seeds did not show any visible signs of toxicity such as stunting, wilting, etc.

The entries of ENPs into seeds are a tough task compared to plant cell walls and membranes due to its thick seed coat. The water transport pathways are responsible for the translocation of ENPs within seeds and plants (Thurman et al., 1972), suggesting the penetration capability of nZnS through seed coat via water transports system that promoted the germination. Also, it was reported that large size aggregates of ENPs had induced toxicity in the seeds by blocking the ion and water channels or apoplastic pathway (Asli and Neumann, 2009). However, as no phytotoxic symptoms were observed in nZnS treated seeds, it can be concluded that the aggregates of nZnS did not induce any physical toxicity in mungbean seeds. Previously, Siddiqui et al., 2014, reported an enhanced seed germination of Lycopersicon esculentum at 8 g L^{-1} concentration of $nSiO_2$ (Siddiqui and Al-Whaibi, 2014). Similarly, Almutairi (2016), reported an enhanced germination of seeds in Lycopersicon esculentum at low concentration of SiNPs. Our result was in correspondence with the earlier report

and suggested that the use of low concentrations of nZnS and minimal uptake by seeds could be one of the reasons to have enhanced seed germination [**Fig. S2**].

Growth profile of the treated plants was also measured in terms of root-shoot lengths, fresh-dry weights and rootlet numbers. In our study no phytotoxic symptoms were observed either in leaf or in root at any treatment concentrations of nZnS. All treated plants were healthy and significant enhancement in growth profile was observed after 10 days of treatment (**Fig. 2A**). As summarized in Table 1, 0.1 mg L^{-1} concentration of nZnS was found to be the most effective among all the applied dosages of nZnS. At this dose, nZnS significantly increased root and shoot length of mungbean plants by 46.84% and 31.38%, respectively over control. Besides, fresh and dry weights (wt.) of nZnS treated plants at 0.1 mg L^{-1} concentration were also increased by 68.59% and 37.67%, respectively. Rootlet number of nZnS treated plants at 0.1 mg L^{-1} concentration was also increased by 31.51%. Previously, Suriyaprabha et al, 2012, showed significant effects of SiNPs on *Zea mays* in hydroponic medium and found that germination rate and growth percentage were enhanced (Suriyaprabha et al., 2012). The direct uptake of ENPs by seeds was improved in a hydroponic incubation that rendered potential barriers for plants and hence beneficial results were obtained. According to Rawat et al. (2019), at optimal concentration and medium term exposure ENPs can be used as a plant growth promoter or fertilizer. Our results were corroborated previous findings and suggested the

positive effect of nZnS on growth of mungbean plants that can be used as plant-nanomodulator in future.

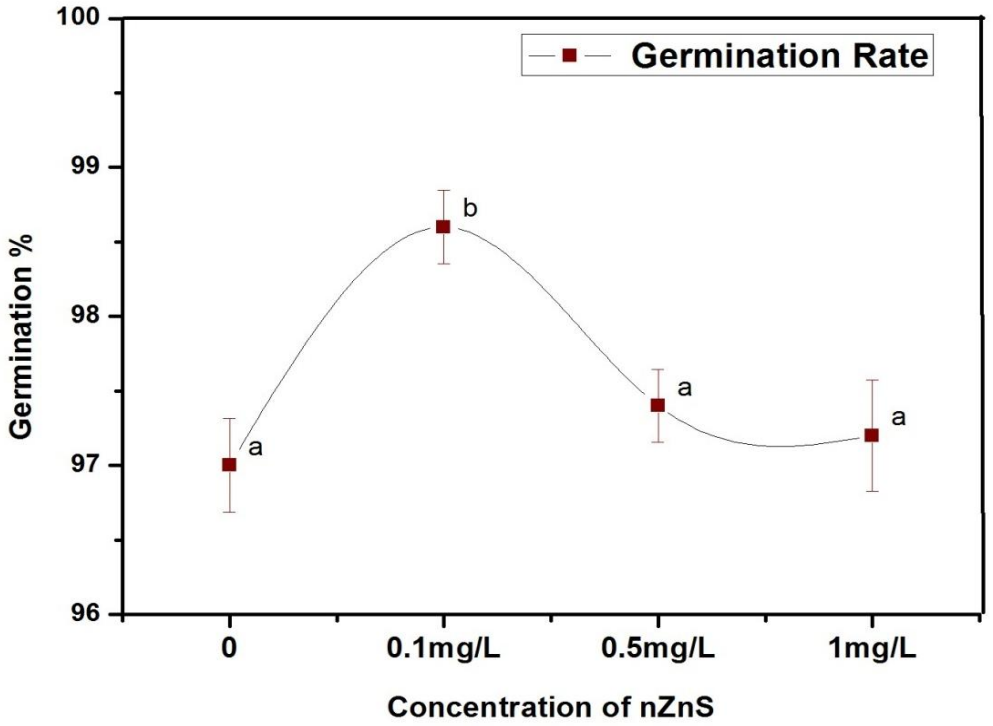


Figure S2. Effect of nZnS on Seed Germination percentage (%) after 24 hrs [Total seed=100]. Values are means ± SE (n=5). Means with the same letter along the column are not significantly different at Tukey's test ($p<0.05$).

Treatment	Control	0.1mg L^{-1} nZnS	0.5mg L^{-1} nZnS	1mg L^{-1} nZnS	F and p Values
Root length(cm)	7.1±0.03 a	10.42±0.230 b	9.79±0.074 c	9.67±0.190 c	F= 90.68, $p \leq 0.001$
Shoot length(cm)	10.2±0.04 a	13.39±0.046 b	12.71±0.08 c	12.67±0.07 c	F= 526.04, $p \leq 0.001$
Fresh weight(mg)	2.7±0.02 a	4.54±0.012 b	3.25±0.029 c	2.95±0.017 d	F= 1736.59, $p \leq 0.001$

Dry weight(mg)	0.2±0.002 a	0.3±0.001 b	0.27±0.001 b	0.26±0.001 b	F= 4.46, $p \leq 0.001$
Rootlet no.	8.2±0.006 a	10.79±0.008 b	10.48±0.024 c	10.42±0.012 c	F= 6804.95, $p \leq 0.001$

Table1. Effect of nZnS on root-shoot length, fresh-dry weight and rootlet numbers of 10 days treated mungbean plants. Values are mean±SE (n=5). Different letters designate significant change at Tukey's test ($p<0.05$).

2.3.2.2. Pigments content: Photosynthesis is a sensitive physiological process. Its efficiency decreases under stress conditions. To determine the effects of nZnS application on photosynthesis, we measured the pigment content of the nZnS treated plants. The results showed that, plants exposed to nZnS had significantly higher pigment content than control. Among all the treatment concentrations, highest chlorophyll content was observed at 0.1 mg L^{-1} concentration, where chlorophyll a (Chl a) was 84.4% and chlorophyll b (Chl b) was 85.7% higher than those of control (Chl a; $F=24.556$, $p\leq0.001$ and Chl b; $F=12.266$, $p\leq0.001$, **Fig. 2B**). Similarly, highest carotenoids content was also found in plants exposed to 0.1 mg L^{-1} nZnS treatment, where carotene was 88.6% and xanthophylls was 86% higher than control (carotene; $F=65.440$, $p\leq0.001$ and xanthophyll; $F=37.012$, $p\leq0.001$, **Fig. 2B**). Previously, Mukherjee et al. (2014), demonstrated that nZnO at 125, 250 and 500 mg L^{-1}, reduced chlorophyll level in peas (*Pisum sativum*). In contrast, Ghafariyan et al. (2013),

reported that in Glycine max chlorophyll level was significantly increased by FeNP treatment at 30–60 ppm concentration. In our study, significant enhancements in both Chl a and b contents in nZnS treated plants over control were observed. As Chl a molecule participates in the photochemical reaction and Chl b on the other hand is accessory pigments that act indirectly in photosynthesis by transferring energy to Chl a, our results suggested that nZnS treatment could enhance plant photosynthate production. Also, as Chl a molecules are directly associated with carbohydrate production, responsible for better growth in vascular plants (Nayan et al., 2016), the growth increment observed in nZnS treated plants could be via increased carbohydrate production. Carotenoids are the light harvesting accessory pigments that absorb light energy and transfer to the chlorophyll molecules and play an important role in protecting the chlorophyll from oxidative damage. Therefore, increased carotenoid contents by nZnS treatment would improve the activity of Electron Transport Chain (ETC) and provide protection from oxidative damage in plants (Pradhan et al., 2013). Overall, enhanced photosynthetic pigment contents in the treated plants suggested that nZnS could play an important role in augmenting plant's photosynthesis.

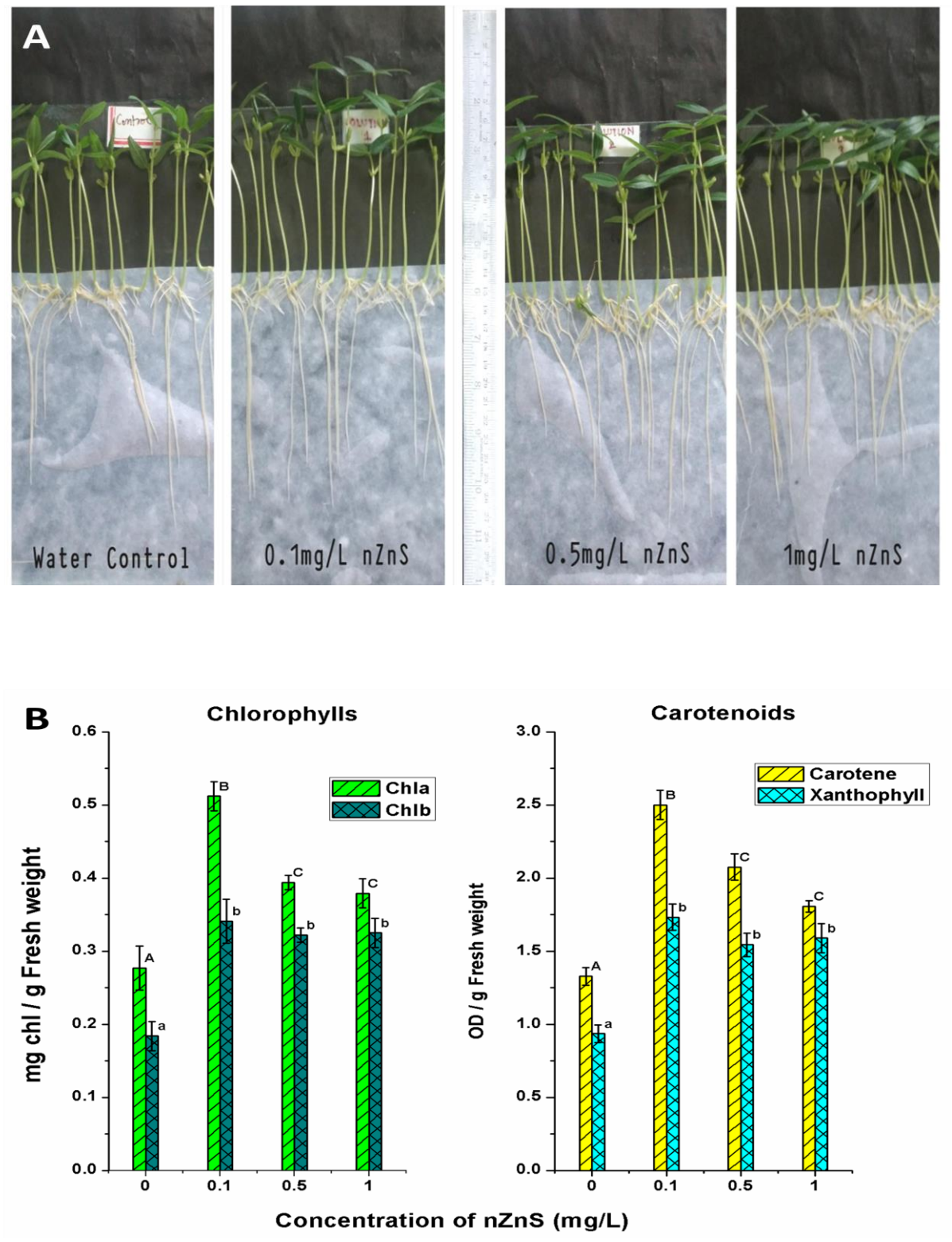


Figure2. (A) Morphology of control and nZnS treated plants after 10 days (B) Effect of nZnS on Chll a and Chll b, Carotene and xanthophyll content of 10 days treated mungbean plants. Different letters designate significant change at Tukey's test ($p<0.05$). Values are means±SE (n=5).

2.3.2.3. H_2O_2 generation, lipid peroxidation, proline, electrolyte leakage and total protein; in light of ROS generation: ENPs generate ROS, which induce lipid peroxidation, membrane leakage etc. and could cause oxidative stress in plants. To test the effects of nZnS on membrane integrity and oxidative stress; H_2O_2 generation, lipid peroxidation, electrolyte leakage, proline and protein content were measured in nZnS treated and control plants. H_2O_2 plays a vital role in plant defense system. At optimal concentrations (4 $\mu molm^{-2}\ s^{-1}$), it acts as a signaling molecule involved in signalling and triggering cellular growth, whereas at relatively higher concentrations (10 $\mu molm^{-2}\ s^{-1}$) it triggers loss of enzymes activity, induces oxidative stress and programmed cell death (Nakano and Asada, 1981). In our study, none of the nZnS treatment doses resulted in any significant accumulation of H_2O_2 as compared to the control (**Fig. 3a**). This result indicated that at the tested concentrations, nZnS did not cause any cellular stress. The recorded H_2O_2 concentrations in treated plants are in the optimal range (1.6–3.15 $\mu M\ gm^{-1}$ fresh weight) indicating that it might be involved in the activation of cellular growth and antioxidant responses.

The enzymatic oxidative product MDA was used as an index to measure the extent of membrane damage caused by ROS generation. Previously, Cabiscol Català et al. (2000), reported that ENPs can affect membrane integrity or permeability and lipid peroxidation by ROS generation. Interestingly, in our study, MDA content were significantly decreased in all the nZnS treatment concentrations than that recorded in the control plants (leaves; F=43.751,

p≤0.001 and roots; F=68.642, p≤0.001, **Fig. 3b**). Results indicated that the nZnS application played a protective role in membrane integrity of plants, presumably due to its ability to induce the anti-stress enzymes (Pullagurala et al., 2018).

Proline is an important stress marker molecule, and plays a role in oxidative stress tolerance in plants. In our study, while no significant change was found in leaves, proline accumulation reduced significantly in the nZnS treated plant roots with respect to control (F=5.067, p≤0.012, **Fig. 3c**). Sharma et al., (2012), have reported a decrease in MDA and proline content in B. juncea seedlings treated with AgNPs. They suggested that the declines in proline level indicate improved electron exchange efficiency in the AgNPs treated seedlings. In line with Sharma et al, 2012, our result also showed enhanced pigment content and root-shoot growth might be due to improved electron exchange efficiency. Also, the reduction in lipid peroxidation by the application of the nZnS supports the proposed use of nZnS as a nanofertilizer in the future (Singh et al., 2013).

Electrolyte leakage is indicative of stress response in intact plant cells and is widely used as a measure of plant stress tolerance (Lutts et al., 1996). Conductivity measurements showed that nZnS treatment resulted in significant increase in root electrolyte leakage (F=9750.33, p≤0.001, **Fig. 3d**). In roots ~9% increase in electrolyte leakage was observed at 0.1 mg L^{-1} nZnS concentration, 10% increase at 0.5 mg L^{-1} and 16% increase at 1 mg L^{-1}. These increased leakages could be the result of roots coming in the direct contact of the particles. When compared to previous studies, leakage observed in our study

was not as dramatic as observed when plants were exposed to AgNPs (at 10 mg L^{-1}) and MWCNT (at 500 mg L^{-1}), which resulted in significant induction of ROS generation and oxidative stress (Oukarroum et al., 2012; De La Torre-Roche et al., 2013). Conversely, electrolyte leakage in leaves of treated seedlings were significantly reduced ($F=3283.54$, $p\leq0.001$). This result was in agreement with the reduction in MDA level indicating protective role of nZnS on membrane integrity (Pullagurala et al., 2018). Overall, nZnS treatments improved cellular electron exchange efficiency in treated seedlings (by maintaining optimum H_2O_2 concentration), arrested electron leakage, and by maintaining the ROS formation it could protect the cell membrane. No significant change in total protein content was observed in treated plants in comparison with the control [**Fig. S3**].

2.3.2.4. Antioxidant defense system: Phenol and flavonoids are low molecular weight plant antioxidants which scavenge free radicals and protect antioxidant system (Ebrahimzadeh et al., 2010). In our study, no significant changes in phenol contents were observed in nZnS treated plants (**Fig. 3e**). However, flavonoid content was significantly increased in all the treated concentrations; the maximum increase was occurred at 0.5 mg L^{-1} treated leaf (leaves: $F=14.91$, $p\leq0.001$ and roots: $F=9.27$, $p\leq0.006$, **Fig. 3f**). Therefore, the increased flavonoids contents in the treated plants highlighted the antioxidant property of nZnS. Total antioxidant activity (TAA) in nZnS treated plants were also

increased significantly than the control plants (leaves; F=1196.52, p≤0.001 and Root; F=35.97, p≤0.001, **Fig. 3g**). Similarly, increased DPPH scavenging activities were observed in treated plants (leaves; F=15615.83, p≤0.001 and roots; F=10937.78, p≤0.001, **Fig. 3h**). Plants showed a concentration dependent response in TAA, with increasing nZnS concentration TAA was also increased in treated leaves. On the other hand, highest DPPH activity was recorded at 0.1 mg L^{-1} nZnS concentration. Likewise, Abdel-Aziz et al, 2014, reported that AgNPs (5 mg L^{-1} to 20 mg L^{-1}) synthesized from *Chenopodium murale* leaf extract showed higher antioxidant and antimicrobial activity compared to C. murale leaf extract alone or silver nitrate. They showed that DPPH values increased in a dose dependent manner (Abdel-Aziz et al., 2014). Similarly, AgNPs synthesized from *Bergenia ciliata* showed higher TAA compared to the plant extract alone (Phull et al., 2016). Therefore, the results of our study demonstrated the antioxidant attributes of nZnS which would help in increasing the overall antioxidant capacity in mungbean plants.

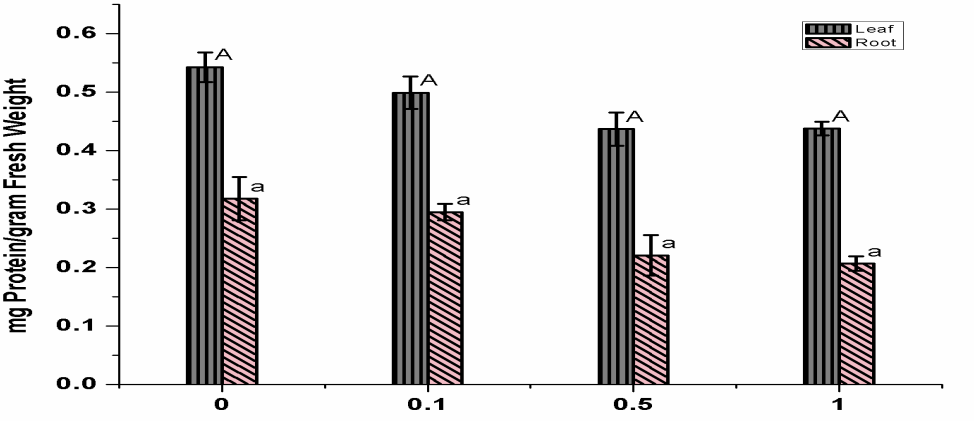


Figure S3. Effect of nZnS on Total Protein content. Values are means ± SE (n=5). Means with the same letter along the column are not significantly different at Tukey's test ($p<0.05$).

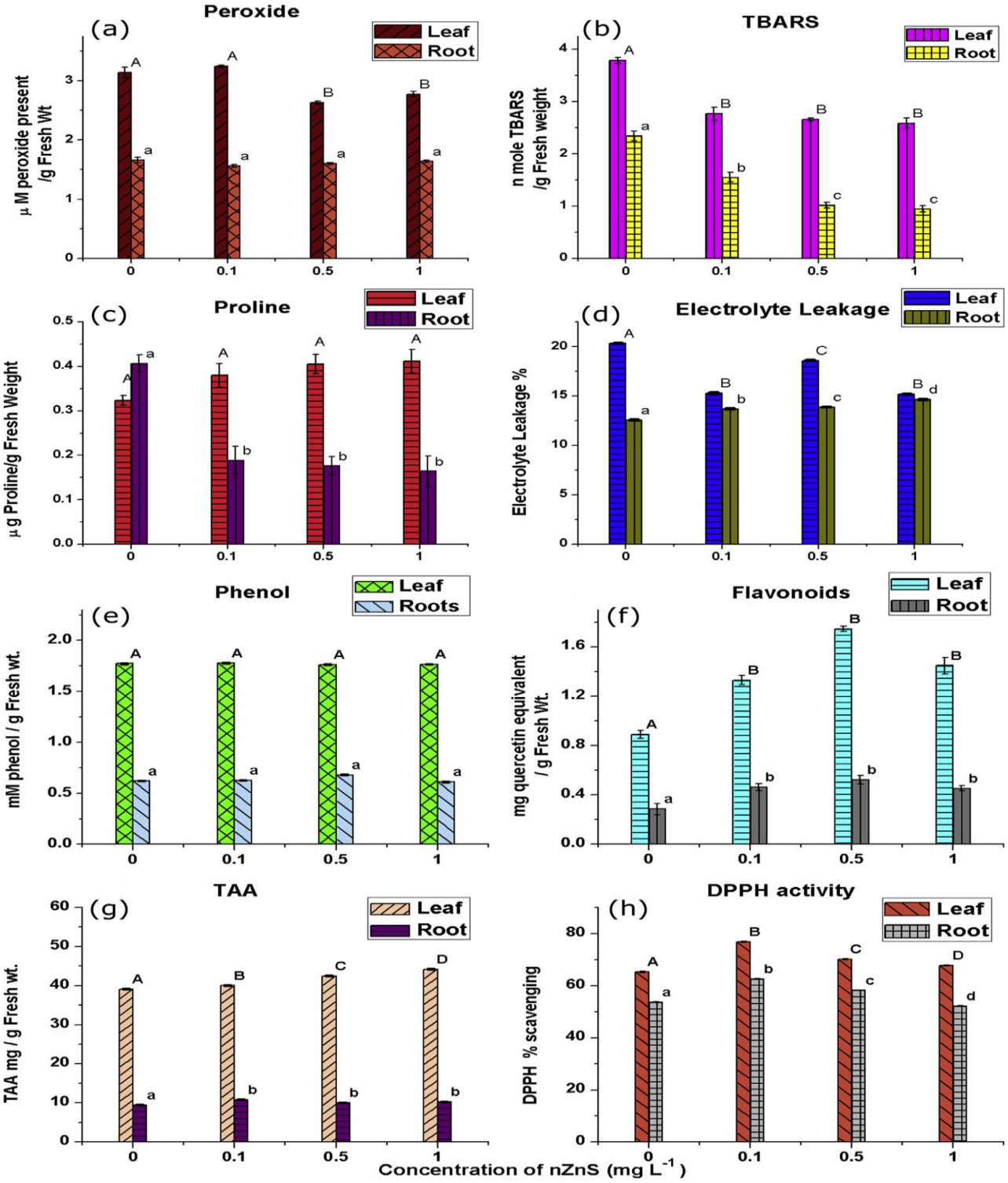


Figure3. (a) Peroxide content (b) TBARS (c) Proline content (d) Electrolyte leakage (e) Total phenol (f) Total flavonoid content (g) TAA and (h) DPPH activity of 10 days treated mungbean plants. of 10 days treated mungbean plants. Different letters designate significant change at Tukey's test ($p<0.05$). Values are means ± SE (n=5).

2.3.2.5. nZnS treated plants did not trigger oxidative stress: To test the effects of nZnS on ROS generation and oxidative stress in mungbean plants, activities of superoxide dismutase (SOD), catalase (CAT), catechol peroxidase (CPX), ascorbate (APOX) and glutathione (GR) were measured. SOD is a powerful stress enzyme that catalyzes the dismutation of O_2^- to H_2O_2. In the present study, change in SOD activity was found only at 0.1 mg L^{-1} nZnS concentration, where significantly decreased activity was recorded in both roots and leaves (3.5% and 4.2% respectively), indicating the protective role of nZnS against oxidative stress (leaves; $F=9.51$, $p\leq0.005$ and roots; $F=8.77$, $p\leq0.007$, **Fig. 4a**). The decreased SOD activity recorded here might be the result of antioxidant activity of nZnS.

In comparison to control, marked increased in root CAT activities (49% for 0.1 mg L^{-1}, 55.39% for 0.5 mg L^{-1} and 79.73% for 1 mg L^{-1} nZnS) were recorded in nZnS treated plants ($F=240.99$, $p\leq0.001$), whereas, activities in leaves decreased (62.46% for 0.1 mg L^{-1}, 54% for 0.5 mg L^{-1} and 35.08% for 1 mg L^{-1} nZnS; $F=95.553$, $p\leq0.001$, **Fig. 4b**). The enhanced root CAT activities, corresponded with the low levels of electrolyte leakage observed in nZnS treated roots, confirmed that the primary organs which come in contact with the

ENPs changed CAT activity that might vary according to the intensity of stress, time of treatment and induction of new isozymes. The plausible reason for the decreased CAT activities in leaves might be due to the existence of certain level of ROS production even in the control seedlings. Thus the regulation of catalase activity must occur by a specific mechanism, not by peroxisomal turnover (Chance and Maehly, 1955).

However, no alteration in the CPX activities were observed in nZnS treated mungbean plants (**Fig. 4c**), a similar trend observed in H_2O_2 generation, and was in agreement with the commonly observed positive correlation between the antioxidant enzymes and stress levels in plants. The APOX is known to have higher affinity towards H_2O_2 than CAT. It is an enzyme in the Halliwell-Asada pathway (ascorbate−glutathione cycle), a network of oxidation-reduction reactions that directly reduces the H_2O_2 generated by SOD into H_2O (**Fig. S4**). Besides, ascorbate peroxidase (APOX) readily dismutes H_2O_2 using ascorbate as the electron donor (Foyer and Halliwell, 1976). As shown in **Fig. 4d**, no significant changes in APOX activities were recorded in both leaves and roots of the treated plants. These results clearly indicated that peroxidase, lipid peroxidation and APOX enzymes activities synergistically corroborated with each other and nZnS treatments did not induce any kind of oxidative stress and maintained cellular homeostasis.

Glutathione reductase (GR) catalyzes the generation of reduced glutathione (GSH) via Halliwell-Asada pathway, needed for the regeneration of ascorbate

(**Fig. S4**) (Foyer and Halliwell, 1976). A significant ($F=219.49$, $p\leq0.001$) increase in GR activities were found in the leaves of nZnS treated seedlings (56.44% for 0.1 mg L^{-1}, 59.6% for 0.5 mg L^{-1} and 67.05% for 1 mg L^{-1} nZnS), however no change was observed in the roots (**Fig. 4e**). Considering that no alteration in APOX activates were recorded, this increase in GSH activities in leaves might be the result of sulfur supplementation via nZnS treatments (Nayan et al., 2016). Previously, Xiang et al, 2001, reported that plants with decreased GR activities were smaller in size and are more sensitive to environmental stresses (Xiang et al., 2001). May et al. (1998), also demonstrated that increased levels of reduced glutathione provided plants with selective advantage to overcome suboptimal growth conditions. Therefore, the increased shoot lengths observed in the treated plants might be the result of increased GR activities. Thus, the results showed that optimum concentration of nZnS triggered improved growth and adaptability in mungbean plants via increased GR activity.

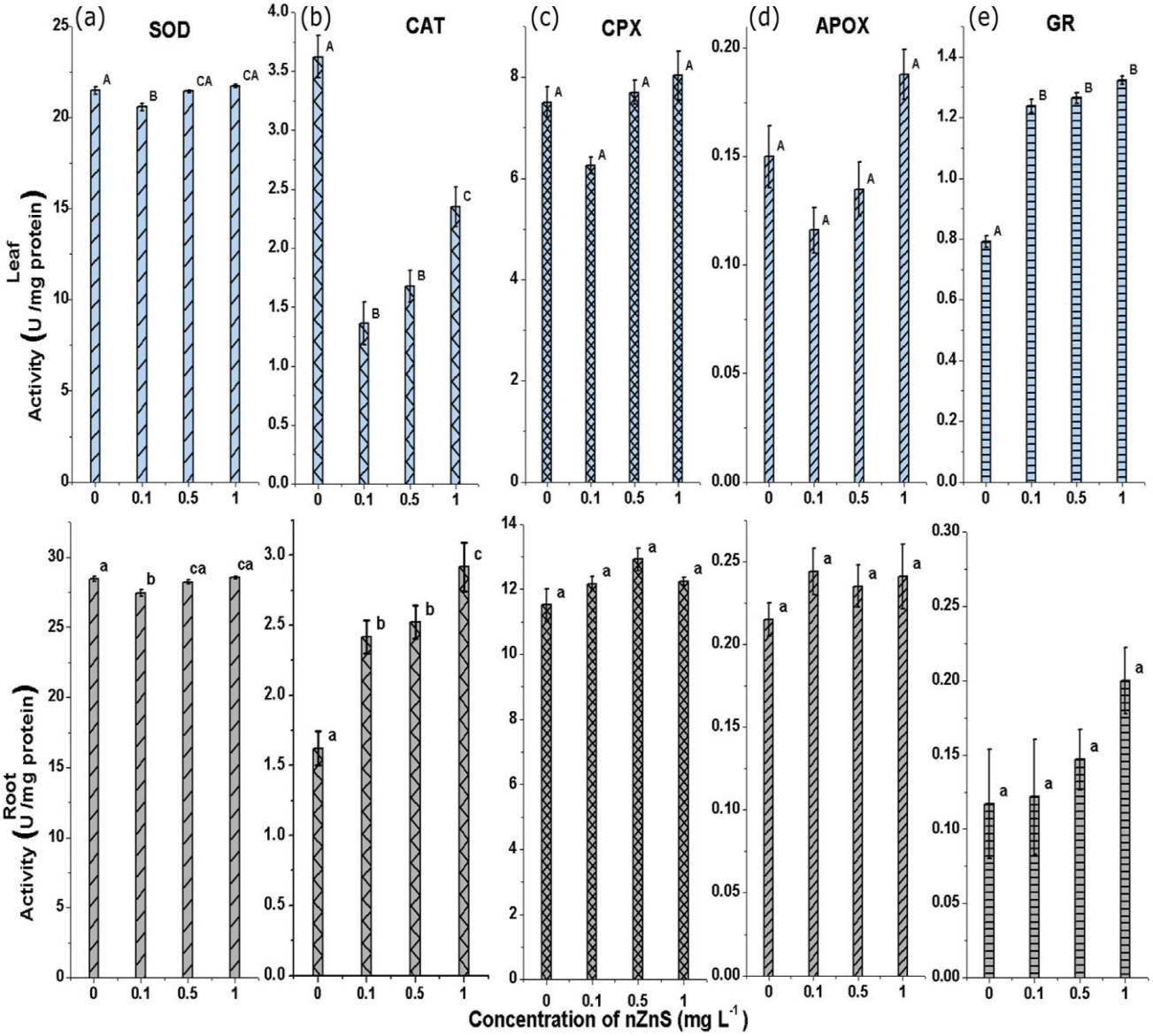


Figure4. Effect of nZnS on the enzymatic activities of (a) SOD (b) CAT (c) CPX (d) APOX and (e) GR. Values are means ± SE (n=5). Different letters designate significant change at Tukey's test ($p<0.05$).

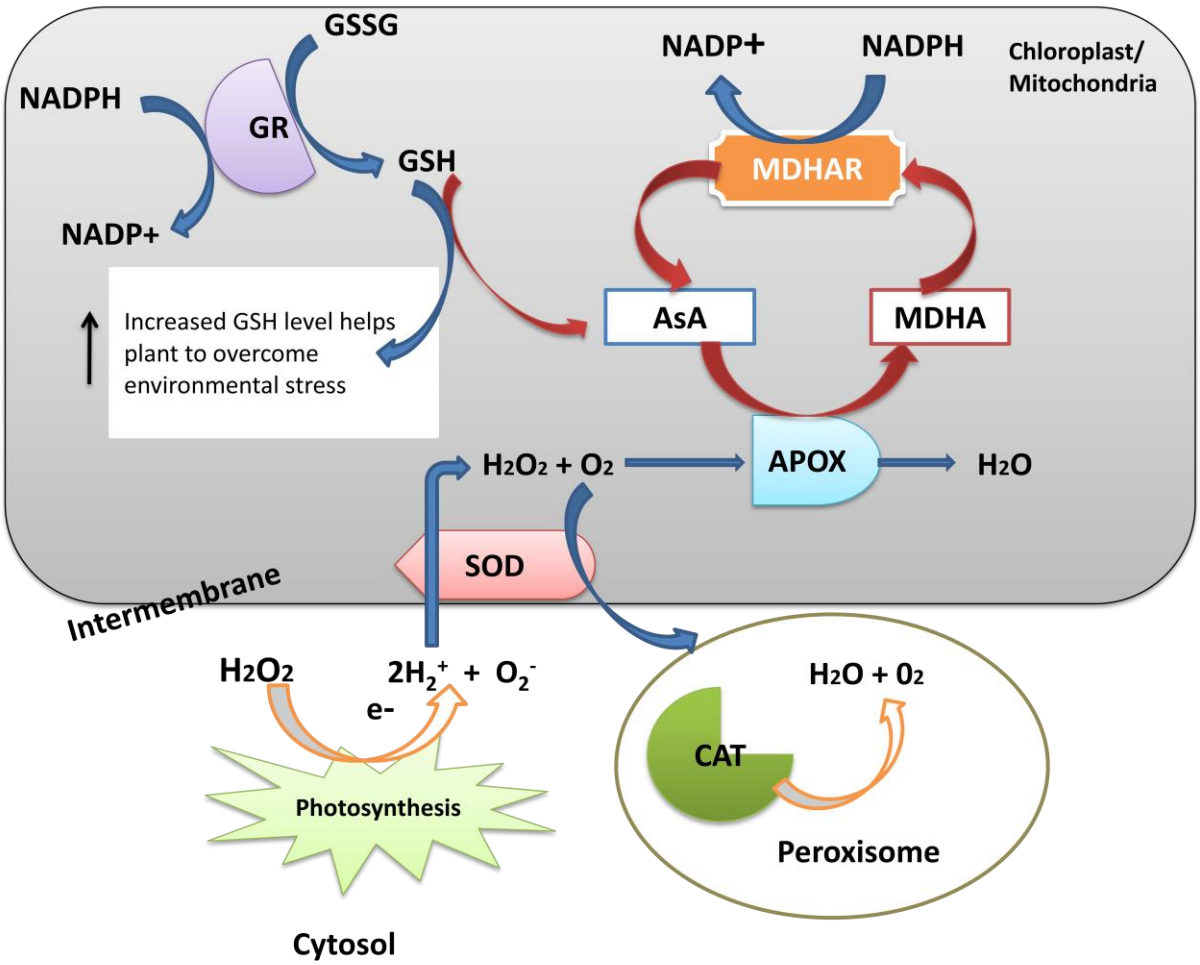


Figure S4. Schematic diagram of the antioxidative system of plants (modified from reference 2). Abbreviations are ascorbate (AsA), glutathione reductase (GR), glutathione reduced (GSH), the oxidized form of glutathione (GSSG), nicotinamide adenine dinucleotide phosphate (NADPH), and oxidized NADPH ($NADP^+$).

2.3.3. Uptake and translocation of nZnS, and its effects on plant cell Microstructure

2.3.3.1. Microscopic evidence of uptake, transport and accumulation of nZnS: nZnS/ionic Zn distribution pattern in cross sections of control and treated plants roots and leaves were obtained using SEM with EDX. SEM images revealed low concentrations of Zn in roots and leaves tissues of treated plants (**Fig. 5**), whereas no Zn was found in control plants. The presence of low concentration of Zn in treated plants suggested possible internalization of nZnS into the plants (Du et al., 2015). In roots, Zn was observed in epidermis, parenchyma, but was not detected in the vascular cylinder which indicated that most of the applied nZnS entered into the root cells and accumulated in the parenchyma region. Similar result was found by Du et al., (2015), who exposed wheat plants to nCeO at 100 and 400 mg kg^{-1}. TEM images further confirmed this information, where nZnS were observed to be present primarily in the vacuoles of the parenchyma cells. Weight percentage of elements and EDX analysis were presented in **Table S1–S5**.

As seen in **Fig. 5**, neither roots nor the leaf tissues of the treated plants showed any structural aberrations and membrane damage. Applications of ENPs often cause structural aberration in plants. For example, Lin and Xing (2008), reported that exposure of nZnO in ryegrass caused shrunk root tips and highly vacuolated root cells. Wang et al. (2011), also showed that ultra-small anatase,

$nTiO_2$ caused dysfunction of microtubules and tubulin monomers in *A. thaliana*. In contrast, nZnS treatment did not generate such phytotoxic responses, while Zn was detected by EDX analysis [**Tables S1–S5**] in leaf midrib proving that nZnS/ionic Zn reached the transport system and was acropetally

translocated from root to leaf. TEM images of treated leaf sections showed accumulation of nZnS in vacuoles and chloroplast regions (**Fig. 6E–H**). However, control plants were devoid of ENPs or aggregates (**Fig. 6A–D**). In treated plant's root section, ENPs were mainly accumulated in the vacuoles (**Fig. 6J**), whereas in control plants no such dark particles were observed in roots (**Fig. 6I**). According to reported studies, particles up to 20 nm were taken up by plant cells through plasmodesmata and endocytosis (Dietz and Herth, 2011). For example, Lin and Xing (2008), used TEM to show that nZnO passed through the epidermis and cortex of roots of *Lolium perenne* L. (ryegrass), but they did not examine if they are present within the shoots. Zhu et al. (2008) used magnetization to show the uptake and subsequent transport of magnetite nFe_3O_4 by *Cucurbita maxima* (pumpkin) grown in solution culture. However, no nFe_3O_4 (i.e., magnetic signals) were detected in shoots of soil cultured plants. Also, Navarro et al. (2012), used polymer coated CdS/ZnS QDs to show uptake by A. thaliana but no internalization and translocation as intact QDs occurred after 7 days of exposure. But in our study, microscopic images evidenced that nZnS could penetrate the root cell wall and translocate acropetally via the water transport system to the leaves without altering cell

structures. The uptake of ENPs by the plants is dependent on many factors; the stability of ENPs in the suspension is one of the important reasons among them. Thus, from environmental point of view investigations on the long term stability of ENPs in different systems and in soil are important.

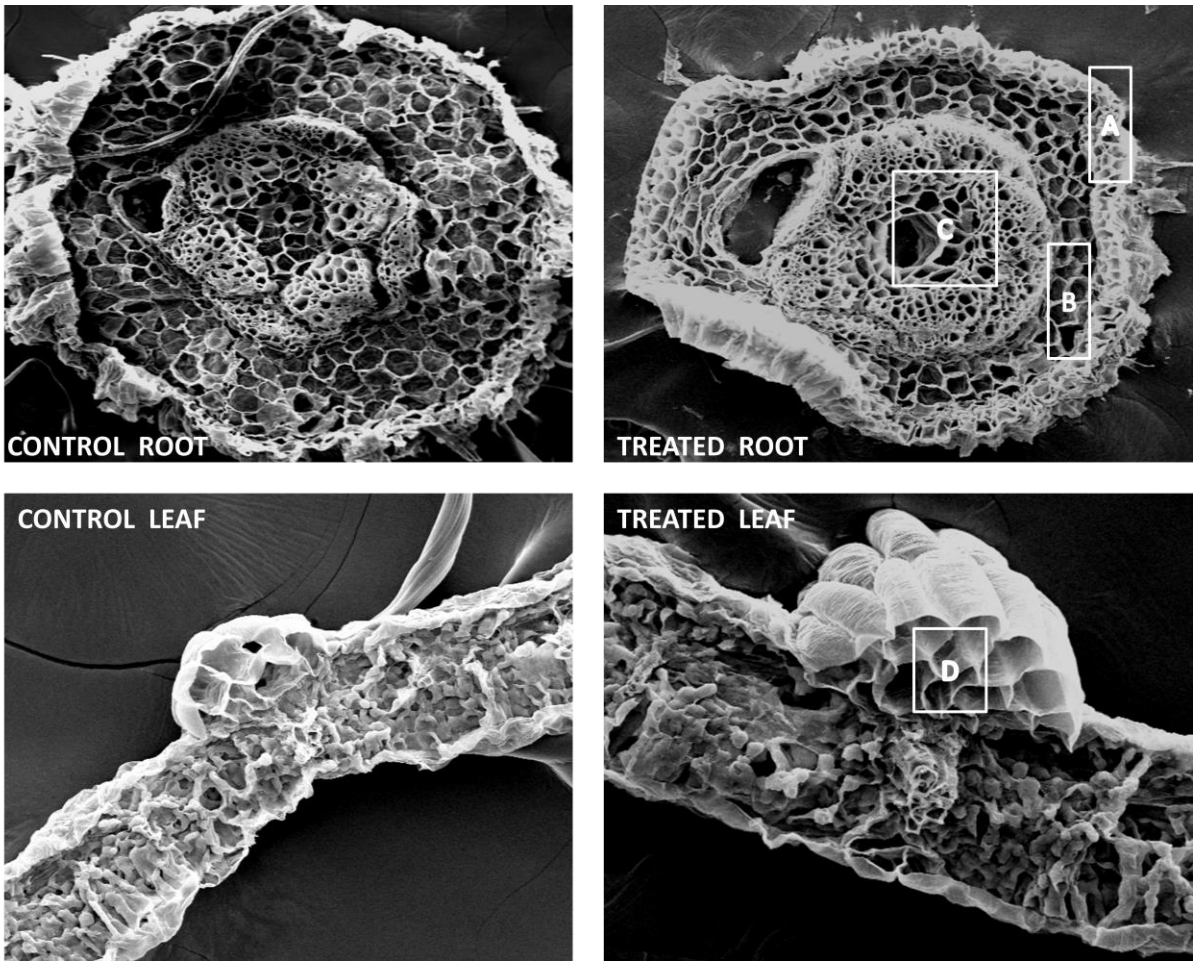
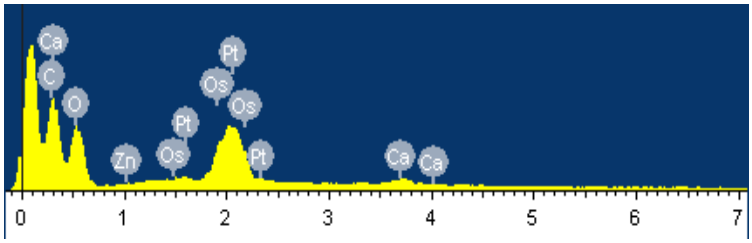
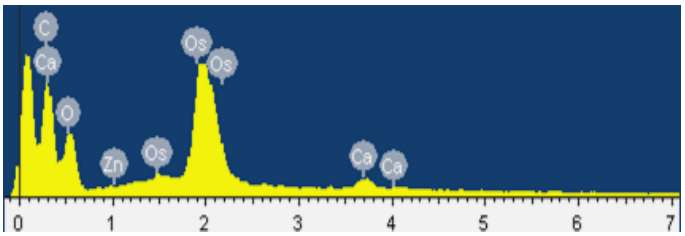


Figure5. Biological SEM image and EDX analysis of cross section from root tips and leaf of control plant and plant treated with 1mg L^{-1} nZnS.(A) Epidermis, (B) Parenchyma (C) Vascular cylinder (D) Leaf midrib.



Element	Weight%	Atomic %
C K	26.20	42.06
O K	3.14	54.64
Ca K	10.36	0.54
Zn K	0.5	0.11
Os M	44.30	1.10
Pt M	15.50	1.55
Totals	100.00	

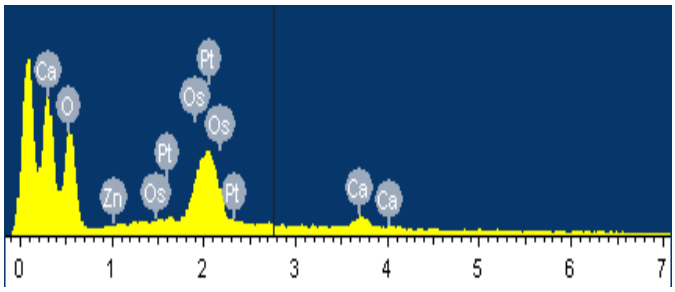
Table S1. EDX analysis of 1mg/L nZnS treated plant root epidermis.



Element	Weight%	Atomic%

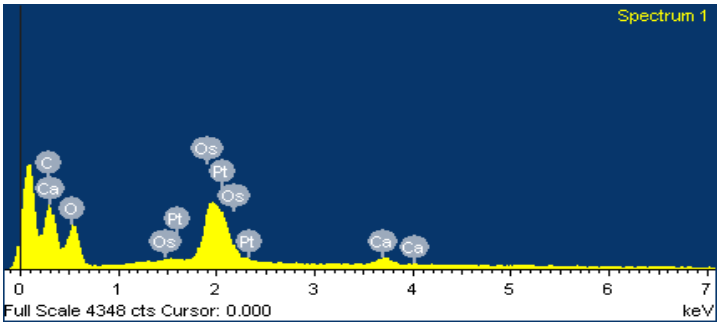
C K	10.40	42.06
O K	4.34	54.64
Ca K	26.30	0.54
Zn K	0.2	0.11
Os M	43.56	1.10
Pt M	15.20	1.55
Totals	100.00	

Table S2. EDX analysis of 1mg/L nZnS treated plant root parenchyma.



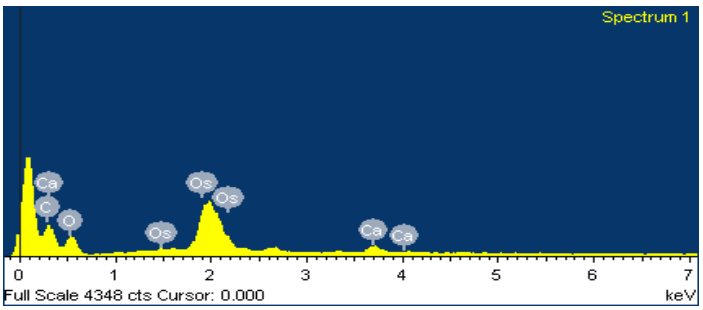
Element	Weight%	Atomic%
O K	1.89	51.90
Ca K	44.44	0.88
Zn K	0.20	0.06
Os M	24.72	2.43
Pt M	28.75	1.55
Totals	100.00	

Table S3. EDX analysis of 1mg/L nZnS treated plant leaf vein.



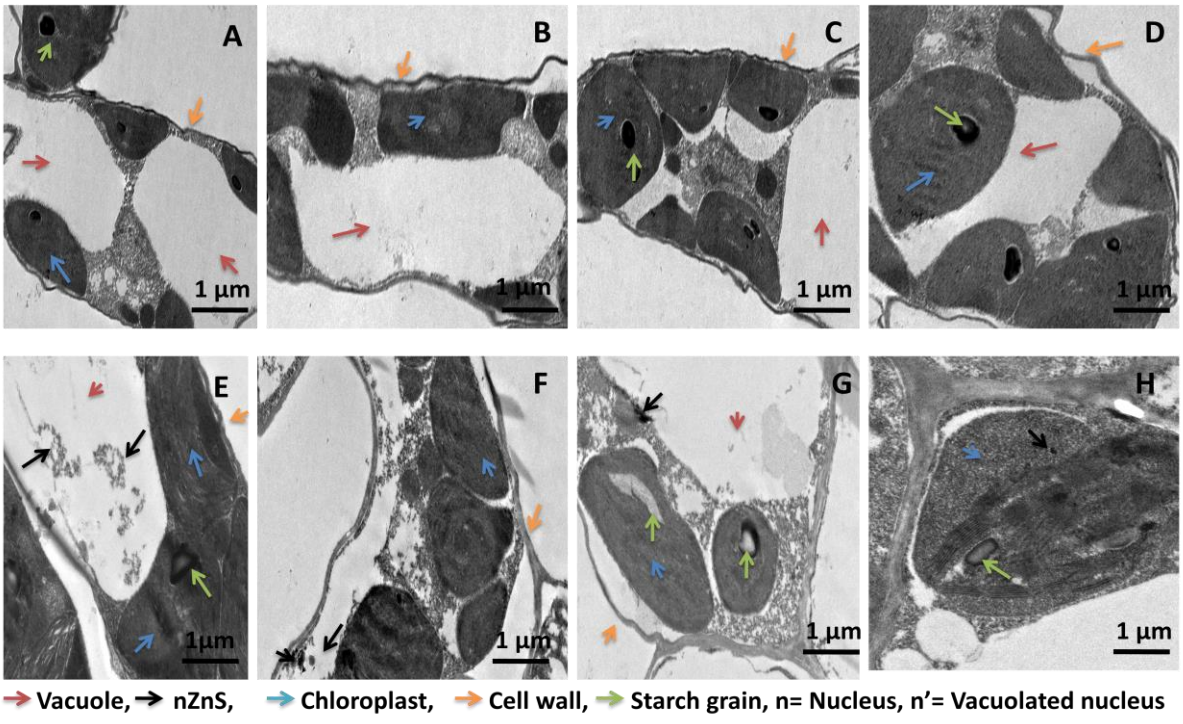
Element	Weight%	Atomic%
C K	24.60	41.52
O K	43.05	54.55
Ca K	1.31	0.66
Os M	17.21	1.83
Pt M	13.83	1.44
Totals	100.00	

Table S4. EDX analysis of root cross section of control plant.



Element	Weight%	Atomic%
C K	24.65	43.40
O K	39.29	51.93
Ca K	1.66	0.87
Os M	20.95	2.33
Pt M	13.45	1.46
Totals	100.00	

Table S5. EDX analysis of leaf cross section of control plant.



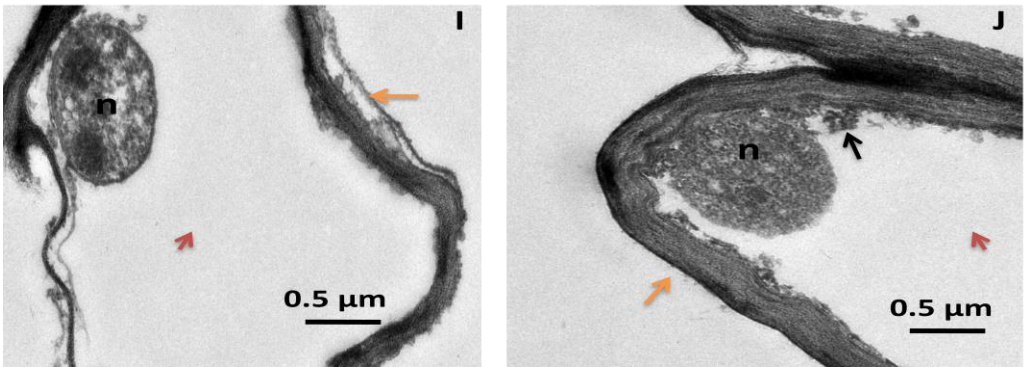


Figure6. TEM images of mungbean plant; leaves (control: A, B, C, D and treated with nZnS: E, F, G, H) and roots (control: I and treated with nZnS: J).

2.3.3.2. Zn release from nZnS: In vitro ICPMS at pH 7.0 studies on release of Zn from treatment concentrations (0.1, 0.5 and 1 mg L^{-1}) of nZnS, revealed that very small amounts of 0.001 ppm, 0.003 ppm, and 0.005 ppm Zn were released, respectively, after 24 h. In vivo study of distribution of nZnS on plant samples was also studied using ICPMS. Results showed that nZnS treated plant samples had small but significant concentration dependent enhancements in Zn contents in both roots ($F=50.460$, $p < 0.001$) and leaves ($F=104.998$, $p < 0.001$) with respect to control [**Fig. 7**]. Results also confirmed the slow release of Zn from nZnS and its uptake by treated plants. The highest concentration of Zn was accumulated in roots than leaves of treated plants due to the direct exposure to the treatment solutions. Also, the dose dependent increase of Zn in treated plant leaves compared to control confirmed the translocation of nZnS from root to leaves.

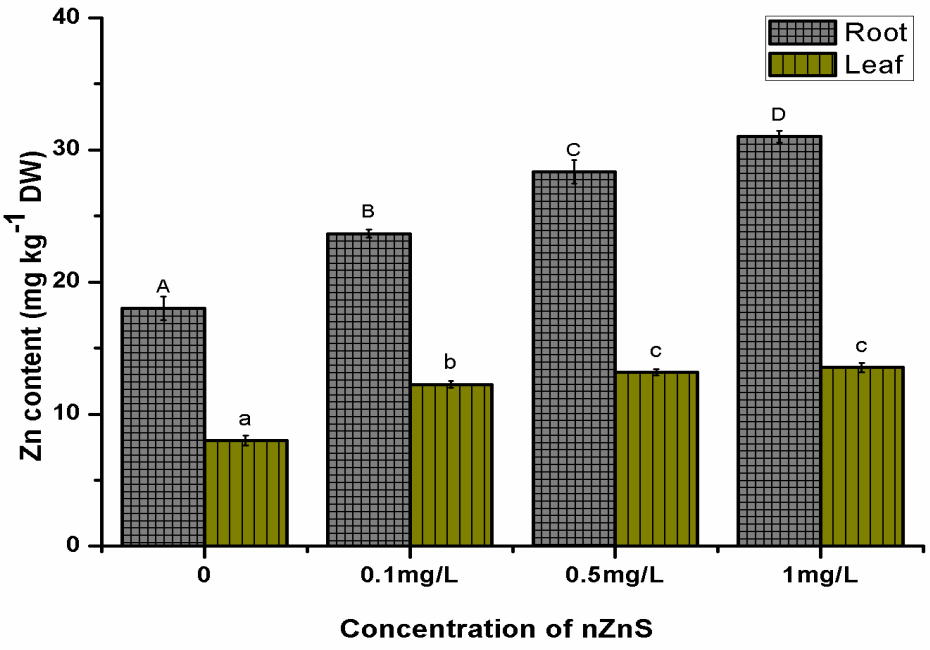


Figure7. Zn accumulation in mungbean plant estimated by ICPMS. Values are means ± SE (n=3). Means with the same letter are not significantly different at Tukey's test, at p≤0.05.

2.3.4. Cytotoxicity Study: ROS is the product of aerobic metabolism in plants, which acts as signalling molecules, whereas excess ROS cause DNA damage, electrolyte leakage, lipid peroxidation and membrane damage, finally causing cell death. There are many reports available on oxidative stress and membrane damage caused by NPs. So we used Evans blue dye as a marker to measure membrane integrity or membrane damage. Living cells have the ability to exclude the dye at the plasma membrane, while cells with a damaged membrane are unable to exclude the dye and are stained blue. As seen in **Fig. 8A**, no

significant increase in Evans blue uptake was observed at all concentrations tested while the positive control (+control) showed a 4 fold increase in Evans blue uptake. The result indicated that the exposure doses of nZnS did not damage root cells and are not toxic to *A. cepa* root cells.

2.3.5. Genotoxicity Study: The genotoxicity of nZnS was further studied in the root meristematic cells of *A. cepa* using the chromosome aberration and micronucleus assays, following 24 hours of nZnS exposure. Chromosomal aberrations were characterized by presence of anaphase/telophase bridges, early/late chromosome separations that are manifestation of spindle aberration, binucleate cells etc. Also, according to Ma et al., micronuclei are the most effective parameter of studying cytological damages resulting from environmental contamination.

In our study exposure of various concentrations of nZnS revealed i. No chromosomal aberrations observed even at highest concentration of 1 mg L^{-1} of nZnS, ii. Most of the cells are in early and late prophase stage, iii. No micronucleus formation was observed, and iv. Normal nuclei with proper nucleus observe at treated sets, (**Fig. 8B**). Similar results reported by Pesnya et al. 2013, where no cytotoxic or genotoxic effects of chitosan-capped silver nanoparticles were observed in *A. cepa* roots at concentrations below 5 mg L^{-1}.

Thus, exposures with 0.1, 0.5 and 1 mg L^{-1} concentrations did not increase the frequencies of chromosomal abnormalities or micronuclei over the control

values. The absence of induction of abnormalities is possibly due to the low concentration and slow release of NPs. A significant increase in the frequency of chromosomal aberrations was observed only in the +control, **Table 2**.

2.3.6. DNA laddering: DNA laddering study was shown in **Fig. 8C**. As shown in the image there was no DNA fragmentation presence in treated *A. cepa* roots with respect to the control. DNA was characterized by the presence of a single prominent band corresponding to its genomic DNA without DNA fragmentation. Results of DNA laddering in *A. cepa* revealed no DNA damage over a 24 hrs period of time exposure. This result corroborated with the genotoxicity results that the nZnS exposure did not induce DNA or chromosomal abnormalities. Here, the dose and time of exposure played a critical role in showing no DNA damage but more genotoxicity study of nZnS with different concentrations and time exposure would be interesting to validate this findings.

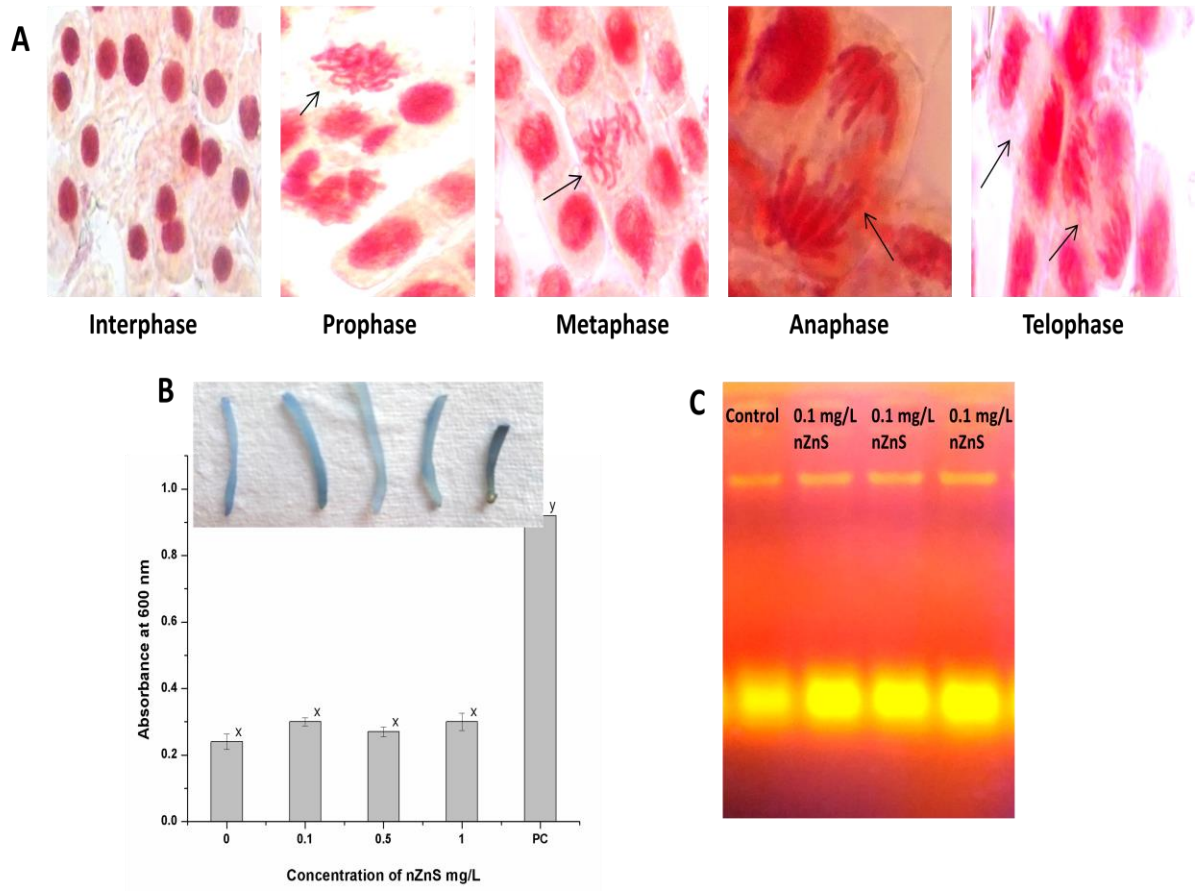


Figure8. (A) Showing prophase, metaphase, anaphase and telophase of 1mg L^{-1} nZnS treated *A. cepa* roots (B) Cytotoxicity (Evans blue dye exclusion assay); Graph representing cytoxicity in *A. cepa*; Figure in inset showing Evans blue uptake by *A. cepa* root cells.Means with the same letter are not significantly different at Tukey's test, F= 179.84,p< 0.001 (B) (C) DNA laddering assay of treated and control *A. cepa* root genomic DNA.

Exposures	**Chromosomal aberrations**				**Chromosomal aberration index (%)**	Micronucleus
	Sticky	Bridge at anaphase	Fragments	c-mitosis		
Control	-	-	-	-	Nil	-
0.1 mg L^{-1}	-	-	-	-	Nil	-
0.5 mg L^{-1}	-	-	-	-	Nil	-
1 mg L^{-1}	-	-	-	-	Nil	-
+control	+	+	+	+	4.6	+

Table2. Chromosomal aberrations and micronuclei index of control and nZnS treated cells of *A. cepa*. (1000 cells were scored for each sample and each exposure groups have 5 samples).

2.3.7. Potential for nZnS to be use as a micronutrient: As revealed by the TEM images, presence of nZnS aggregates, mostly in vacuoles, suggested that nZnS moved from xylem's sap to aerial tissues with apoplastic flow (through cell wall) and symplastic transport (through cytoplasm). Further studies will be required to validate this finding. Also, the low biomass distribution of Zn in the tissues indicated the low dissolution rate of nZnS, which remained in nano form after 10 days of treatment. The particles which were found in TEM analysis primarily had a diameter of 20 ± 0.2 nm. However, the average size of the applied particles was 13.3 ± 0.3 nm. This could be explained by the fact that nZnS formed agglomerates in the cell medium, which were slightly larger than the initial particle sizes. The released Zn together with nZnS was carried through to the leaf of the plant samples interacted with microenvironment of

treated plant cells and augmented pigment level and plant growth, in contrast with the control. Therefore, nZnS application has the potential to correct Zn level in crops. Again, photosynthesis is a well established source of ROS in plants. Superoxide and H_2O_2 are generated by photosynthetic components on Photosystem I (PSI). A balance must be maintained between ROS generation and scavenging in plant system. Importance of the antioxidants in maintaining photosynthesis has been reported earlier by many researchers (for example, see Pradhan et al., 2013). In this context, our study demonstrated that the applied nZnS positively altered plant growth performance and antioxidant status. Increased chlorophyll content, the optimum level of H_2O_2 and superoxide molecules, and enhanced antioxidant capacity in nZnS treated plants highlighted the nZnS antioxidant machinery that might act in PSI and regulate cyclic electron flow to limit singlet oxygen production at PhotosystemII (PSII).

Also, the significant reduction in lipid peroxidation by nZnS treatment favours the proposed use of nZnS as a nanofertilizer in the future and a detail mechanistic study on reason behind this will be interesting. Again, high antioxidant capacity in nZnS treated plants is beneficial because it desensitizes photosynthesis to overreduction in the Photosynthetic Electron Transport chain. Thus present study demonstrated the potential beneficial role of nZnS in altering metabolic pathways of mungbean plant but a life cycle study is also necessary.

2.4 Conclusion

Our study evidenced that nZnS treatments promoted root-shoot lengths and produced higher photosynthetic pigments in treated plants. The biochemical assays conducted during the study evidenced that among the tested doses of nZnS, 0.1 mg L^{-1} was optimal for inducing maximal growth stimulatory responses. None of the treatments triggered any oxidative stress but improved antioxidant system of the treated mungbean plants. The electron microscope studies confirmed uptake and translocation of nZnS in treated plants and also showed that it did not cause any damage to the cellular microstructure.

This study showed that nZnS did not exhibit phytotoxicity, cytotoxicity and genotoxicity effect on plants at different concentrations. The total chromosomal abnormalities and micronuclei were not affected by nZnS exposure. Intact genomic DNA was observed in treated *A. cepa* roots with respect to the control.

Therefore, given the growth promotion effects of nZnS treatment in plants, it can be concluding that it has the potential to be used as a micronutrient for plant growth and photosynthetic enhancements in plants. While further studies will be necessary before nZnS could be recommended for field use, the results generated in our study provided preliminary information about the novel plant-modulatory roles of nZnS.

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CHAPTER 3

Application of Zinc Sulfide Nanoparticles to Augment the Nutritional Status of the Mungbean [*Vigna radiata* (L.) R. Wilczek] Plant

3.1. Introduction

In recent years, a wide range of applications of nanoparticles (NPs) in agriculture, food industries, drug delivery, etc., has increased the chances of NPs release into the environment, prompting researchers to undertake studies of the interactions of NPs with living organisms and environment.[1] Plants play a pivotal role in the transportation of NPs in the ecosystem, through uptake and bioaccumulation, and are often used as indicator systems for an assessment of toxicity.[2] Plant responses to NPs vary from negative to inconsequential to positive depending on the plant species, growth medium, concentrations, and types of NPs studied.[3] Many studies have reported that the application of NPs improved crop productivity by enhancing the germination, seedling growth, biomass accumulation, and physiological activities including photosynthesis, nitrogen metabolism, fatty acids contents, etc.[4] Conversely, toxicity responses in plants treated with NPs also have been reported.[5] The toxicity of NPs to plants are known to be the result of the generation of reactive oxygen species (ROS) inducing oxidative stress, a shading effect, and agglomeration of NPs.[4b,6]

Intracellular signaling for the activation of a plant defense system against foreign particles is mediated by a lipid-based signal transduction cascade. Fatty acids are important cellular components that act as energy sources, required for membrane integrity as well for cell proliferation. Saturated fatty acids (SFAs) are more stable than unsaturated fatty acids (UFAs). UFAs maintain membrane

fluidity and are susceptible to rancidity. A series of enzymes called fatty acid desaturases (FADs), evolved in the production of UFAs.[7] In addition, UFAs are essential nutrients for humans, especially polyunsaturated fatty acids (PUFAs), such as ω-3 linolenic acid (18:3) and ω-6 linoleic acid (18:2), required in several physiological processes. But humans do not have the necessary enzymes to convert ω-6 into ω-3 because of the absence of ω-3 FADs;[8] therefore, one must obtain these from the diet. Because of the significance of PUFAs as nutrition and their role in cellular processes, the industrial production of PUFAs has received great attention. In fact, an NP-induced plant-based synthesis of lipids or UFAs has been reported by a few researchers. For example, increased production of lipids and UFAs was found in *Pichia pastoris* by $nTiO_2$ treatment.[9] In *Chlorella vulgaris*, treatments of nano Cu, Zn, Pb, and Mg promoted a production of UFAs.[10] In groundnut, nano Se treatment increased the UFA production,4c and also increased UFAs were found in *Scenedesmus obliquus* due to nFe_2O_3, nMgO, and carbon nanotubes (CNTs) treatments.[11] Thus, the development of crop plants with a health-beneficial proportion of ω-3 and ω-6 fatty acids by NPs treatments is important.[12] A variety of plant-based functional foods has been recommended by health experts worldwide, encouraging changes in the diet, in order to improve human health and prevent chronic diseases.[13] Legumes are the second most consumable food in the world after cereals. Mungbean (*V. radiata*) is a major edible legume crop in Asia and is also consumed in Southern Europe and in the Southern United States. It is a

good source of nutrients like protein, dietary fiber, minerals, vitamins, and antioxidants, such as phenols, which provide health benefits.[14] As mungbean is easily accessible and affordable to lower-income populations, it is a major source of nutrients for poor people around the world and has been referred to as the "poor man's meat".[15] The aim of the majority of legume improvement programs was to enhance the productivity by using a varietal selection. However, very little attention has been given to the nutritional quality of the crop.[16] Therefore, to meet the increasing demand for legumes, both yield and nutrition needed to be improved.

Zinc sulfide nanoparticles (nZnS) are an important semiconductor with a wider band gap than zinc oxide nanoparticles (nZnO).[17] However, nZnS have not been investigated in detail relative to nZnO.[17] Many efforts have been made toward the synthesis of ZnS nanostructures in different sizes and shapes as well as for specific use in biological detection and tagging molecules.[18] It is widely used in cosmetic, pharmaceutical, and rubber industries.[17] It is preferred in industries due to its simple synthesis techniques, low production cost, large surface area, and high-throughput fabrication of materials for different applications. However, an assessment of its effects in living organisms or the environment has only received limited attention.[18] Hence, its interaction with plants and related mechanisms remain to be elucidated. Moreover, Zn is crucial for a plant's biomass production. It plays a key role in diverse physiological and metabolic processes, for example, chlorophyll and carbohydrate formation,

germination, growth regulation, etc. But most of the Zn in soils is present in unavailable forms, leading to a Zn deficiency, which causes an improper functioning of various metabolic and physiological processes in crop plants. Soils with high pH or sand are responsible for the low availability of Zn. Traditional agriculture practices use Zn sulfate ($ZnSO_4$) or an ethylenediaminetetraacetic acid (EDTA)-Zn chelate as a source of Zn; however, the efficiency is low.[19] Therefore, it is noteworthy to see if nZnS could be used as a nanofertilizer in agriculture. Also, our earlier report on hydroponically grown mungbean (*V. radiata*) plants demonstrated that nZnS treatment improved the antioxidant status and reduced lipid peroxidation.[20] However, there is still a gap in our understanding of the effects of nZnS in the nutritional quality of the crop. Therefore, the present study evaluated the effect of nZnS on (a) nutritional status: (i) plant growth, (ii) fatty acid compositions, and (iii) micronutrients contents of the mungbean plants; followed by (b) its phytotoxicity (if any).

3.2 Materials and Methods

3.2.1. Synthesis and Characterization of nZnS: A detailed synthesis and physicochemical characterization of nZnS was presented in chapter 2.[20] Briefly, nZnS was prepared by a modified reflux method. Fifty milliliters of zinc nitrate (1 M) was taken and refluxed under nitrogen atmosphere at 80 °C. Then, 50 mL of sodium sulfide was added in a drop-by-drop fashion to the above solution and stirred for 6 h. A thick white precipitate thus obtained was centrifuged at 10, 000 rpm for 10 min. The precipitate was washed several times and vacuum-dried to obtain an nZnS powder. The synthesized particles were spherical in shape and less than or equal to 20 nm in size.

3.2.2 nZnS Suspension Preparation and Experimental Design: The suspensions of nZnS at three different concentrations, that is, 0.1, 0.5, and 1 mg/L, were prepared by dissolving nZnS powder in double-distilled water (ddH_2O) and were sonicated before every application.[20] Mungbean [*V. radiata* (L.) R. Wilczek] seeds were collected from the Pulse and Oil seed Research Station, Berhampur, West Bengal, India. Seeds were surface-sterilized with 5% sodium hypochloride solution (w/v) for 10 min and subsequently washed several times with ddH_2O. Seeds were then immersed in different concentrations of nZnS suspensions (control, 0.1, 0.5, and 1 mg/L) and kept in the dark for 4 h. DDH_2O was used as the control. After imbibation, seeds were

kept in Petri dishes lined with filter paper moist with the respective treatment suspensions for germination. After the germination, seedlings were transplanted in plastic cups (20 seeds/cup) filled with natural soil, amended with respective treatment suspensions. The pristine Zn content in the soil was 0.75 mg Zn/kg, and the physicochemical properties of the soil were given in the **Table S1**. Seedlings were grown for 10 d at room temperature (28° ± 2 °C) and in a 14 h photoperiod (light intensity 440 μmol/m2/s). The experiment was performed in a completely randomized design with three replications for each treatment.

3.2.3. Plant Growth: The effects of nZnS on plant growth were studied in terms of root−shoot lengths and fresh−dry weights. After 10 d of growth, plants were washed thoroughly under tap water followed by washing with ddH_2O. The roots and shoots were separated from the seedlings, and their lengths were measured. The excess water was soaked with blotting paper, and fresh weights were determined. Dry weights were measured after they were dried at 80°C for 24 h.

3.2.4. Determination of Reducing Sugar Content: To prepare plant extracts, 100 mg of dry leaves powder and 5 mL of ddH_2O were taken in a beaker and boiled for 15 min at 100° C. After the boiling, it was allowed to cool to the room temperature. After the cooling, centrifugation was done at 10,000 rpm for 10 min. After the centrifugation, the supernatant was collected and stored at 4°

C. Total reducing sugar content was determined based on the 3,5-dinitrosalicylic acid (DNS) method.[21] Briefly, 1 mL of the plant extracts was mixed with 2 mL of the DNS reagent and boiled for 5 min. After the samples were allowed to cool down to the room temperature, the absorbance was determined at 540 nm. The reducing sugar content was calculated from the calibration curve of standard D glucose, and the final result was expressed as mg glucose equivalent/ mL liquid.[2]

3.2.5. Determination of Total Lipid: Total lipids were measured according to Bligh and Dyer's method with modification.[23] Briefly, 1 g of plant tissues was ground with 1 mL of a chloroform/methanol (1:2) solution and homogenized well. After the homogenization, another 2 mL of a chloroform/methanol (2:1) solution was added to the homogenate and mixed well. The mixture was then centrifuged at 3000 rpm for 10 min, and the supernatant was transferred to a new glass tube with a stopper. Three milliliters of a chloroform/methanol (1:2) solution and 0.8 mL of 1% KCl were added to the pellet and vortexed well. The mixture was then centrifuged again at 3000 rpm for 10 min, and the supernatant was separated in the previous glass tube. Following which, 2 mL of chloroform and 1.2 mL of 1% KCl were added to the collected supernatant and vortexed well. After centrifugation at 3000 rpm for 5 min, the lower layer (lipid extract) was transferred to another glass tube with a Pasteur pipet. The remaining chloroform was evaporated by nitrogen stream. The tubes with lipid

were then weighted, and weights of total lipid were calculated with the following formula.

$$Lipid = \frac{W_{c+l} - W_c}{W_{sample}}$$

Where,

W_c = weight of empty tube

W_{c+l} = weight of tube with lipids

W_{sample} = weight of sample

3.2.6. Fatty Acid Profiling: For fatty acid profiling, 1 g of plant tissues (root and leaves) was extracted with 1 mL of methanol and centrifuged at 10 000 rpm for 10 min. After the centrifugation, the supernatant was collected and filtered through a 0.45 μ syringe filter and stored at 4° C for a high-performance thin-layer chromatography (HPTLC) (CAMAG-Anchrom) analysis. Standards of ~1 mg of linoleic, linolenic, palmitic, stearic, and lauric acid with 1 mL of methanol each were vortexed until the material was completely dissolved. The standard solutions were filtered through a 0.45 μ syringe filter and kept at 4° C for further use.[24] For quantification, 5 μL of both standards and samples were added in HPTLC plates in a band-wise fashion. For the mobile phase, n-hexane and ethyl acetate (5:4 v/v) were used. The temperature was kept at 25° C, and the mobile phase was developed in a twin trough glass chamber. After the

development the plates were dried, and an evaluation was performed at 200 and 450 nm. For a calibration curve different concentrations (1−50 mg/mL) of standards were used.

3.2.7. Effects of nZnS on Micronutrients Accumulation: Plant samples (root and leaves) from each treatment were dried and digested in a microwave-accelerated reaction system using a mixture of plasma pure HNO_3 and H_2O_2 (1:4).[25] The digested solutions were then filtered through Whatman filter paper (Grade 40, 150 mm) and used for the estimation of zinc (Zn), copper (Cu), iron (Fe), and manganese (Mn) contents. Micronutrient contents were quantified using Atomic Emission Spectroscopy (Agilent Technology 4210, MPAES) and expressed as milligrams per gram dry weight.

3.2.8. Light Microscope Study: At harvest, roots and leaves of control and treated plants were thoroughly washed with ddH_2O, cut in thin transverse sections (T.S.), and fixed with a 2% glutaraldehyde solution at 4° C for 2 h. This was followed by postfixing the samples for 2 h with a 1% osmium tetroxide solution. The samples were then dehydrated with graded ethanol and observed under a light microscope (LM, Nikon EclipseE600).[26]

3.2.9. Statistical analysis: The data were expressed as mean ± standard error (SE) of three replicates. Statistical differences among treatments were

determined using a one-way analysis of variance (ANOVA) followed by a Tukey's test at a significance level of 0.05.

3.3 Results and Discussions

3.3.1. Effect of nZnS on Plant Growth. Figure 1 represented the healthy morphology of 10 d control and treated plants. No visible toxicity symptoms were recorded rather for all the treatment doses of nZnS, both root ($F = 21.00$, $p < 0.001$) and shoot lengths ($F = 27.95$, $p < 0.001$) were significantly increased compared to the control (**Figure 2a**). The average root and shoot lengths of the control plants were 7.5 ± 0.3 and 10.5 ± 0.28 cm, respectively, and the highest increase in root (12 ± 0.3 cm) and shoot (14.75 ± 0.25 cm) length was recorded at the 0.1 mg/L nZnS concentration. Similarly, the fresh ($F = 11.37$, $p = 0.003$) and dry ($F = 11.61$, $p = 0.003$) weights of treated plants were significantly higher in comparison to the control, with the highest weight recorded at 0.1 mg/L nZnS concentration (**Figure 2b**). Results from our study corroborate a number of previous findings that showed that NP treatments improved plant growth and biomass accumulation. For example, Raliya et al. (2015), reported a significant increase in the plant biomass, root−shoot growth, and root area in *Solanum lycopersicum* with the exposure of nZnO; Faizan et al. (2018) reported an increased growth and photosynthetic efficiency by nZnO in tomato plants; and Nayan et al. (2016) reported an increased dry weight, chlorophyll content, and sugar accumulation in *Brassica juncea* by nZnS.[27] In another study, Zhao et al. (2013) reported that the root biomass was not affected by applications

of nZnO at 400 and 800 mg/kg concentrations on cucumber.[28] Similarly, Zhang et al. (2015) reported that nZnO treatment did not show any negative impact on Zea mays.[29] The results of our study showed that nZnS exposure positively changed root−shoot lengths and fresh−dry weights of the treated plants and did not hamper the plant morphology. Hence, in this study the nZnS application improved plant growth and biomass.

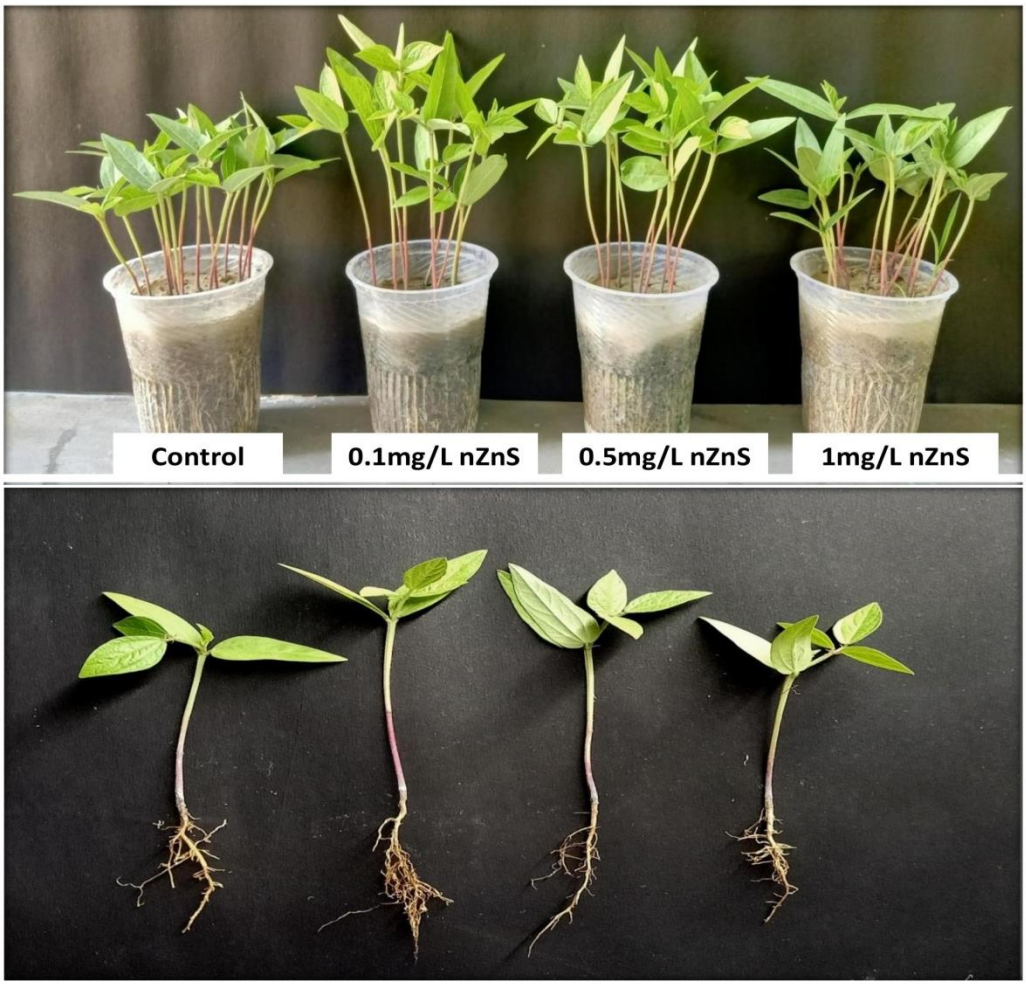

Figure1: Morphology of 10 days old control and nZnS treated plants.

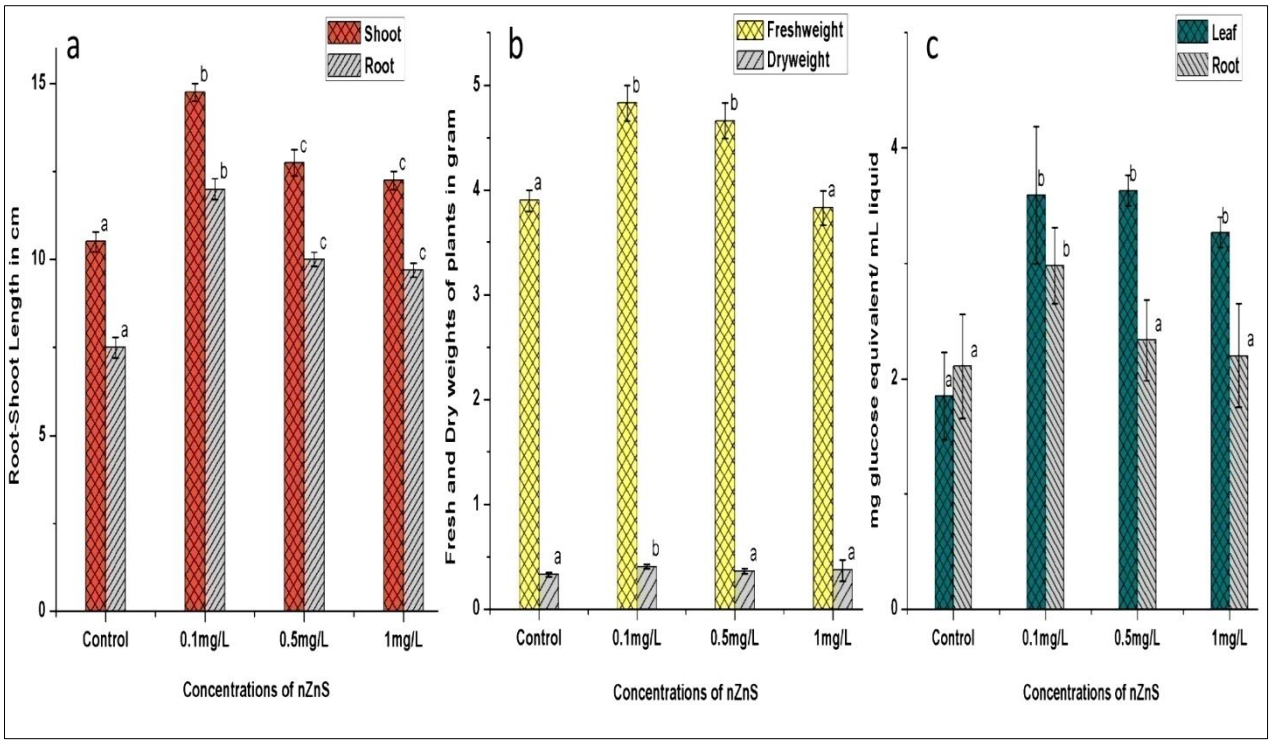

Figure2: Effect of nZnS on (a) root-shoot length, (b) fresh and dry weights and (c) reducing sugar content. Values are means ± SE (n=3). Means with the same letter are not significantly different at Tukey's test (at p <0.05).

3.3.2. Effect of nZnS on Reducing Sugar Content: Similar to plant growth, nZnS applications also had significant effects on total reducing sugar contents in both roots ($F = 14.741$, $p = 0.001$) and leaves ($F = 31.25$, $p < 0.001$) of treated plants as compared to the control (**Figure 2c**). Overall, nZnS treatments increased total reducing sugar contents in leaves and roots across all the treatments, with the highest increase recorded at 0.1 and 0.5 mg/L treatment concentrations. Our results corroborated the increased root−shoot lengths of treated plants and indicated a possible mechanism for positive plant growth. Sugar is the final product of photosynthesis, and due to its active participation in

plant growth, development, storage, signalling, and stress acclimation, it is regarded as the key molecule in plant life.[30] Soluble sugars occupy a central position in the cellular redox balance through their close relationships with photosynthesis, mitochondrial respiration, and fatty acid β-oxidation.[31] During photosynthesis, carbon is assimilated in the form of a carbohydrate, and an increased level of sugar content indicates improved carbon assimilation and better growth.[27b] Hence, our results suggest that increased sugar contents in treated plants could be responsible for an increased plant growth and biomass. Therefore, our study showed positive effects of nZnS treatment on mungbean plants and may have a role in various plant metabolic pathways.

3.3.3. Effect of nZnS on Lipid and Fatty Acids Profiling: The results showed a significant increase in total lipids in both roots ($F = 13.80$, $p = 0.002$) and leaves ($F = 249.58$, $p < 0.001$) of treated plants relative to that of the control (**Figure 3a**). The highest increases in the total lipid content were found at 0.1 and 0.5 mg/L nZnS treatments. No significant difference was found between 0.1 and 0.5 mg/L nZnS-treated plants roots and leaves. Previous reports showed that NP treatments could hasten a lipid production in treated plants. For instance, total lipid content was increased in n$MgSO_4$-treated *Chlorella vulgaris*.[32] Also, both neutral and total lipid contents were increased in *Scenedesmus obliquus* in the presence of CNTs, nFe_2O_3, and nMgO.[11] In

agreement with the previous reports, our study showed that nZnS treatments stimulated a lipid production in mungbean plants.

Results also showed significant differences in the UFAs and SFAs content, except for the palmitic acid contents across all the treatment concentrations relative to the control. The highest increase in linoleic acid content was 88% in leaves ($F = 370.99$, $p < 0.001$) and 28% in roots ($F = 1284.92$, $p < 0.001$) recorded at 0.1 mg/L treatment concentration (**Figure 3b**). Similarly, the linolenic acid content increased by 77% in leaves ($F = 482.23$, $p < 0.001$) and by 11% in roots ($F = 66.76$, $p < 0.001$) at 0.1 mg/L nZnS concentration over the control (**Figure 3c**). The retardation factor (Rf) values of standard linoleic and linolenic acids were found to be 0.67 and 0.7, which corroborated previous reports.[24,33] The specificity of fatty acids was confirmed by comparing Rf values of standards and samples (**Figure 4a-a′,b-b′**). In previous studies, increased PUFA contents in membrane lipids, in particular, linolenic acid, were reported in many plant species due to a low temperature that correlated with a higher level of phosphatidyl choline and ethanolamine, predominantly esterified with linolenic acid.[34] An increase in PUFAs has been thought to increase the membrane fluidity, whereas the decrease in membrane fluidity under stress probably causes a loss in the membrane permeability.[35,36] Hence, PUFAs, which are synthesized by a series of FADs, determine the physical properties of the cell membrane. Furthermore, ω-3 and ω-6 are essential fatty acids needed for cellular functions and growth. In plants, the proportion of linoleic and linolenic

acids vary significantly between species.[37] Moreover, linolenic acid is a precursor of some of the most important long-chain PUFAs, specifically, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which cannot be synthesized in a human.[38] Humans are dependent on plant-derived food for linolenic acid. Again, long-chain PUFAs are important for maintaining the cellular membrane by regulating cholesterol and eicosanoid synthesis, and higher intakes of PUFAs have been shown to lower the risk of coronary artery disease, other cardiovascular outcomes, and certain other chronic diseases.[39] Therefore, the increased linoleic and linolenic acid contents in plants suggest a positive role of nZnS treatment on nutritional qualities of the mungbean plants.

In contrast to the above results, no change was found in the palmitic acid content (**Figure 3d**). Interestingly, significant reductions in lauric and stearic acids contents were recorded in nZnS-treated plants with respect to the control. The highest reduction in lauric acid ($F = 558.63$, $p < 0.001$) content was 18% in the leaves of plants treated with 0.1 mg/L nZnS (**Figure 3e**). However, no significant change in root lauric acid content was found. Significant decreases in stearic acid content were found in both the roots ($F = 231.4$, $p < 0.001$) and leaves ($F = 235.3$, $p < 0.001$) of plants treated with 0.1 mg/L nZnS. The biggest decreases in stearic acid contents were 35% in the leaves and 14% in the roots, relative to the control **(Figure 3f**). The Rf values of standard lauric and stearic acids were found to be 0.8 and 0.06 (**Figure 4c-c′,d-d′**). In a recent study, nano Se treatment at 20 and 40 ppm concentrations on groundnut plants decreased

saturated fatty acids (palmitic, stearic, arachidic, and lignoseric acids) contents and increased UFAs.[4c] Similarly, Yu et al. (2015) showed that $nTiO_2$ treatment promoted UFAs production and an upregulation of many UFAs-related genes in *Pichia pastoris* to fight against oxidative stress.[9] Our results agree with the previous studies and confirm the positive effects of nZnS on the production of UFAs and the reduction of SFAs. Also, our study was designed according to some previous studies, where NP treatments enhanced plant growth and nutrition and produced fruits with improved nutritional values. For instance, Souza et al. (2019) reported a greater capability in the roots, stems, and leaves of Fe_3O_4−NP-treated common bean plants to take up the nutrients from the soil suggested a beneficial effect of Fe_3O_4−NP for plant development and health.[40] Mahmoud et al. (2019) reported maximum vegetative growth, leaf pigments, and root quality in red radish plants treated with ZnO + FeO NPs.[41] In our study, the growth and nutritional values of mungbean seedlings increased with the nZnS application. Therefore, we hypothesize that treated plants with enhanced growth and nutrition will produce fruits with improved nutritional values. We hope our work will provide a blueprint to better utilize nZnS in food products to improve human nutrition and further encourage advancement in this field.

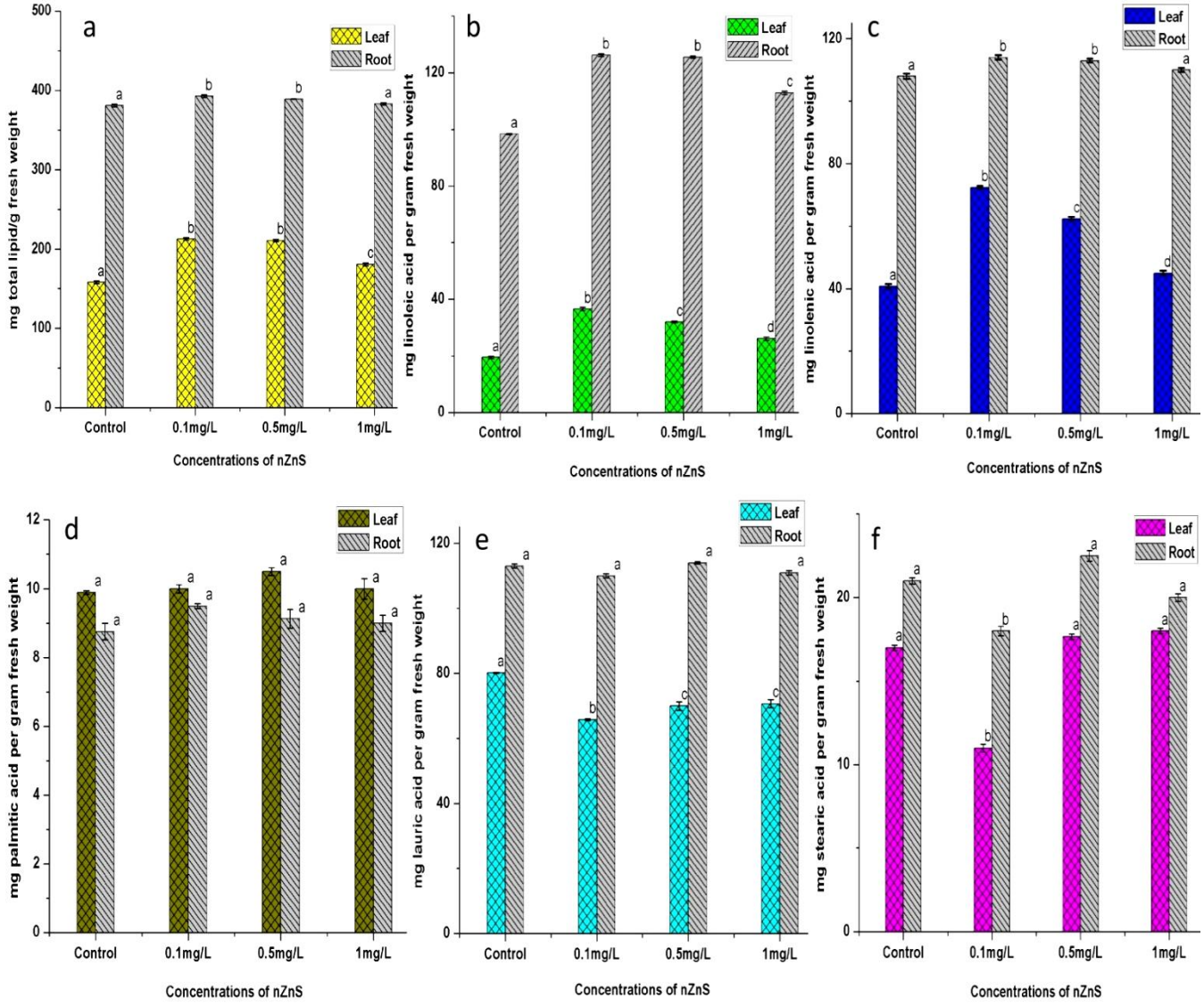

Figure3: Effect of nZnS on (a) total lipid content, (b) linoleic acid, (c) linolenic acid, (d) palmitic acid, (e) lauric acid and (f) stearic acid of 10 days control and treated plants. Values are means ± SE (n=3). Means with the same letter are not significantly different at Tukey's test (at p <0.05).

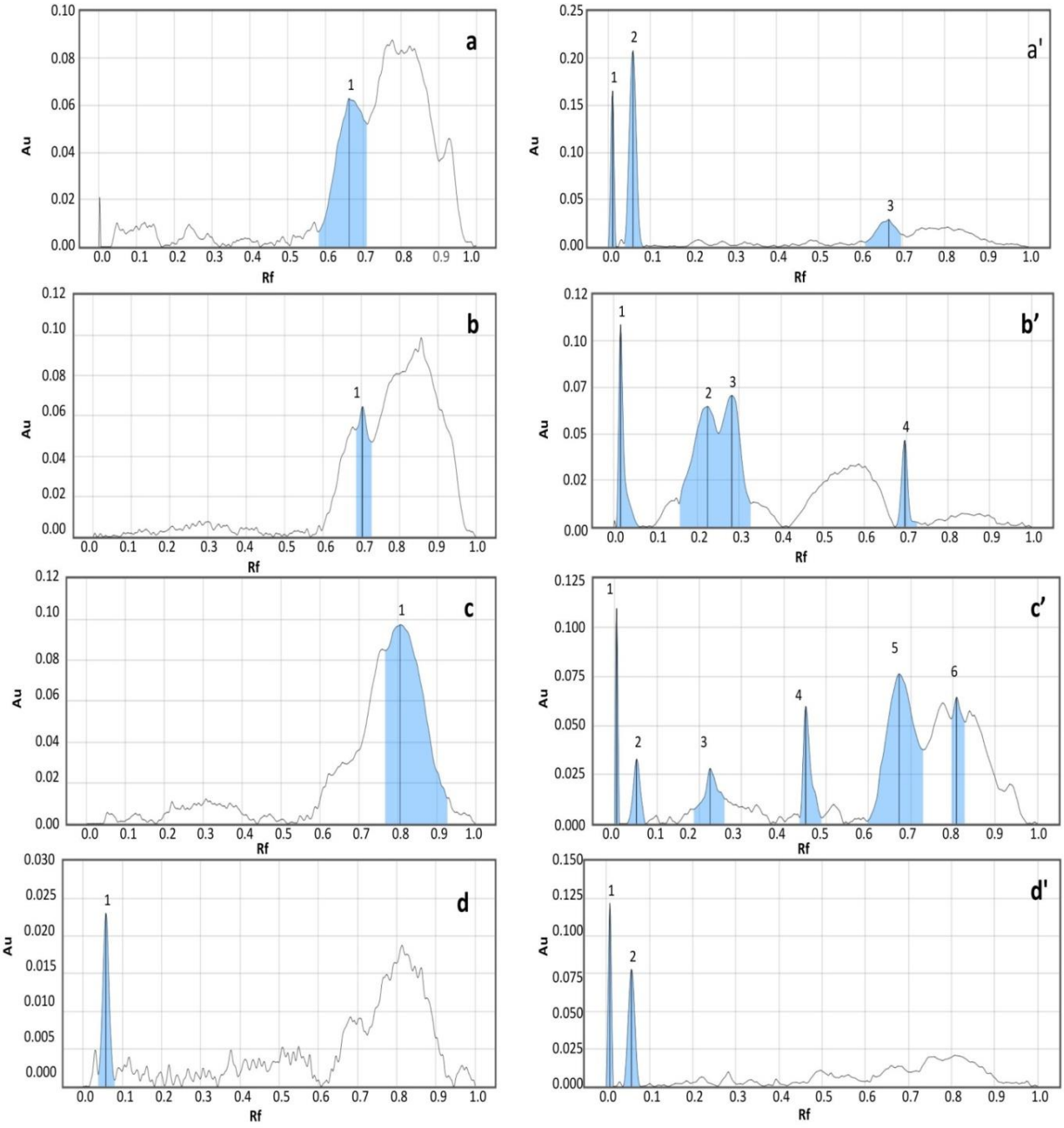

Figure4: HPTLC chromatogram of (a) standard linoleic acid, (a') nZnS treated linoleic acid, (b) standard linolenic acid, (b') nZnS treated linolenic acid (c) standard lauric acid, (c') nZnS treated lauric acid, (d) standard stearic acid and (d') nZnS treated stearic acid.

3.3.4. Effects of nZnS on Micronutrients Accumulation: In our study, nZnS treatments caused a significant increase ($p < 0.05$) in the Zn, Fe, and Mn accumulation in both roots and leaves of the treated plants compared to the control **(Table1)**. The increases in the micronutrient contents were found to be nZnS concentration-dependent. The addition of nZnS treatments resulted in a greater uptake and accumulation of Zn compared to other micronutrients. This increase in Zn varied from 51% to 69% in leaves and from 49% to 75% in roots across treatments relative to the control, whereas the increase in Fe varied from 47% to 66% in leaves and from 32% to 70% in roots, and that of Mn varied from 20% to 53% in leaves and from 38% to 61% in roots. However, no significant difference was found in Cu content. Soil-available Zn was deficient when the value was less than 1 mg Zn/kg, sufficient at 1−2 mg Zn/kg, and excessive at greater than 7.5 mg Zn/kg. Therefore, a Zn fertilizer recommendation could be done according to the sufficiency and deficiency indices of the soil.[42] In our study, the soil was Zn-deficient. Thus, an increase in the Zn uptake by treated plants indicates the potential role of nZnS as a Zn fertilizer. Although micronutrients are essential for the healthy functioning of the body, an excess intake could lead to the generation of damaging radicals. In our study, the highest Zn accumulation found was 0.71 mg/g (leaves) and 0.99 mg/g (roots), the highest Fe accumulation found was 0.035 mg/g (leaves) and 0.053 mg/g (roots), and the highest Mn accumulation was 0.287 mg/g (leaves) and 0.35 mg/g (roots) in the 1 mg/L nZnS treatment. For humans, there is a

limit for micronutrients intake. The recommended dietary intake of Zn for adults is 8−10 mg Zn/d.[43] And the recommended dietary intake of Fe and Mn is 8−11 and 1.9−2.3 mg/d, respectively.[43] Therefore, in this study, the increases in micronutrients contents were in typical serving sizes (optimal level). Moreover, no change was found in the Cu content, probably due to the higher concentrations of Zn in the soil by nZnS treatments inhibiting the Cu uptake by plants (and vice versa) because of a competition for the same sites for absorption into roots.[44] On the contrary, Zn and Fe contents in nZnS-treated plants were reciprocal. Previously it was reported that a deficiency in Zn also led to a deficiency in Fe, due to prevention of the transfer of Fe from root to shoot under Zn deficient conditions.[45] Studies had also shown that a transfer of Fe from the root to shoot depended on the Zn concentration in the medium. In the nutrient medium with minimum Zn, a low level of Fe was transferred from roots to shoot, compared to the same plants that were grown in a nutrient medium with more active zinc.[46] In line with the previous reports, our study also showed synergistic effects of Zn and Fe accumulation in treated seedlings.

Previously, Imtiaz et al. (2003) reported that higher Zn rates significantly reduced Mn concentrations in wheat. They found that the Mn uptake was effected by the presence of different concentrations of Zn in the first growth stage; the uptake of Mn increased up to 10 μg/mL concentrations of Zn, but after that it decreased.[47] In contrast, our study showed a significant increase in

the Mn accumulation at all the treatment concentrations, which is an exception to the trend reported in the aforementioned research. Similar to our study, Soltangheisi et al. (2014) reported that Mn and Zn concentrations in roots and shoots increased with increasing Mn and Zn treatment concentrations in the nutrient medium. The Zn concentration in both roots and shoots was enhanced with increasing Mn levels. The Mn concentration in shoots did not show any correlation with the Zn concentration in a nutrient solution, but the Mn concentration in roots decreased with increasing levels of Zn.[48] Similarly, an application of nFe_2O_3 was found to increase Fe, Mg, Ca, and P contents in Glycin max L. from 0 to 0.75 g/L nFe2O2 concentrations and decreased from 0.75 to 1 g/L nFe_2O_2.[49] Hence, there are still contradictory results present in micronutrient accumulation, which needs further research. Therefore, our study confirmed that ZnS treatments positively affect the micronutrient accumulation in mungbean plants, but further studies will be needed to validate this finding.

Treatments	Zinc (Zn) mg g^{-1} dry weight		Copper (Cu) mg g^{-1} dry weight		Iron (Fe) mg g^{-1} dry weight		Manganese (Mn) mg g^{-1} dry weight	
	Leaf (F= 125.990, p<0.001)	**Root (F= 126.997, p<0.001)**	**Leaf (F= 10.185, p= 0.004)**	**Root (F= 74.228, p<0.001)**	**Leaf (F=12.413, p=0.002)**	**Root (F= 14.696, p=0.001)**	**Leaf (F=23.816, p<0.001)**	**Root (F=57.721, p<0.001)**
Control	0.420±0.01a	0.567±0.06a	0.073±0.03a	0.10±0.06a	0.021±0.02a	0.031±0.16a	0.187±0.16a	0.217±0.3a
0.1mg/L nZnS	0.637±0.02b	0.850±0.02b	0.05±0.28a	0.07±0.16a	0.031±0.01b	0.041±0.02b	0.225±0.15b	0.3±0.20b
0.5mg/L nZnS	0.70±0.01c	0.967±0.01c	0.081±0.16a	0.11±0.05a	0.032±0.03b	0.043±0.16b	0.24±0.20bc	0.33±0.01b
1mg/L nZnS	0.71±0.01c	0.99±0.06c	0.081±0.106a	0.1±0.03a	0.035±0.03b	0.053±0.01b	0.287±0.30c	0.35±0.01c

Table1: Effect of nZnS on micronutrient contents of 10days old plants. Values are mean ± SE (n=3). Letter changes along the column depict significant difference. Same letter are not significantly different at Tukey's test (at p <0.05).

3.3.5. Phytotoxicity Study: The ultrastructures of the leaves of treated and control plants were studied to determine the possible phytotoxic effects of nZnS treatments. Light microscope image of the nZnS-treated leaf showed no alteration in the cellular morphology across all the treatments. No deformities were recorded in mesophyll, palisade, and spongy parenchyma cells, as well as in stomatal structure as compared to the control leaf (**Figure 5a,b**). Available information on NPs toxicity suggested that the NPs sometimes form large agglomerates within the cell that can block the transport system and damage the cellular ultrastructure. Also, the overproduction of reactive oxygen species (ROS) within the plant due to an exposure of NPs might be the primary reason

behind the toxicity of NPs. Again, few studies have shown that phytotoxicity increased with NPs size. For example, a nano Ag exposure had no effect on the growth of castor, but an exposure to bulk Ag resulted in a growth inhibition.[50] Alkhatib et al. (2019), reported that light microscopy images of roots of *Nicotiana tabacum* treated with different sizes (10 and 20 nm) of nFe_3O_4 showed no visible deformation in their ultrastructure in comparison with the control.[51] In our study, due to the small particle size and stability, the nZnS exposure did not show any damage to plant cells and confirmed that the selected concentration of nZnS did not produce phytotoxic responses in mungbean plants.

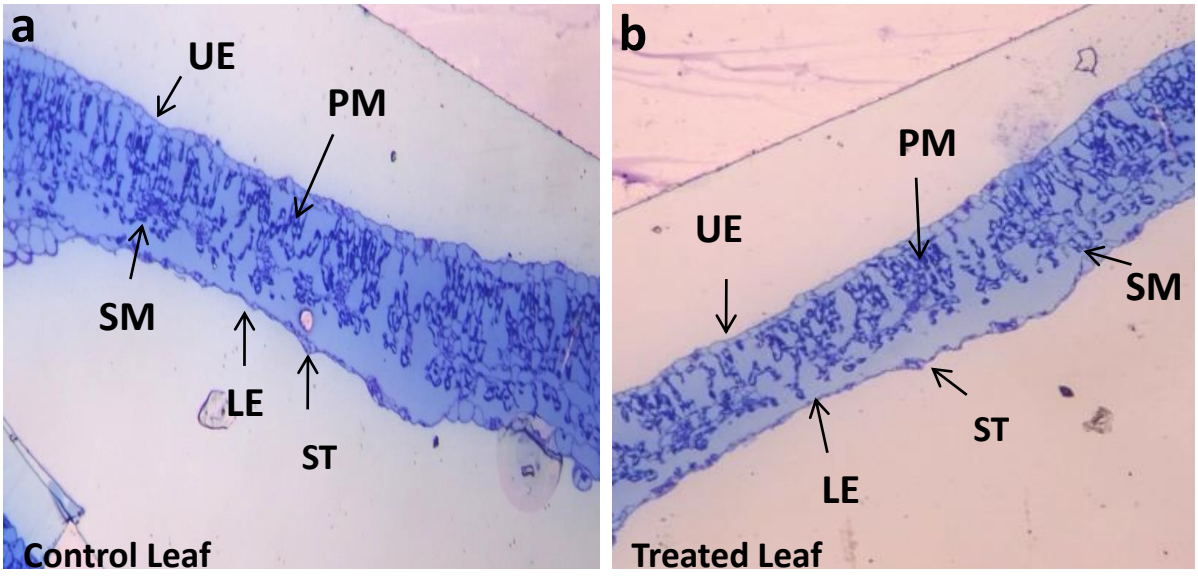

Figure5: Cross sectional light microscopic images of (a) control leaf sample and (b) 1 mg/LnZnS treated leaf sample. EP: epidermis, CT: cortex, VS: vascular cylinder, UE:

upper epidermis, PM: palisade mesophyll, SM: spongy mesophyll, LE: lower epidermis and ST: stomata.

Properties	Particulars	Values
Mechanical composition	Sand (%)	47.6
	Silt (%)	31.5
	Clay (%)	20.6
Chemical properties	Soil pH	7.59
	Organic carbon (%)	0.48
	Available nitrogen (kg/ha)	201.0
	Available phosphorous (kg/ha)	13.5
	Available potassium (kg/ha)	183.8

Table S1: Physico-chemical properties of the experimental soil (0-15 cm soil depth).

3.4. Conclusion

In summary, nZnS treatments significantly increased plant growth, biomass, and total reducing sugar contents in the treated plants. As carbohydrate is the main product of photosynthesis, increased sugar contents by nZnS application could be one of the reasons behind increased plant growth. No phytotoxicity responses were observed in the treated plants.

Interestingly, at experimental concentrations, nZnS was found to be a promising candidate for improving the lipid and UFA production in the mungbean plants. An upregulation of UFAs and a downregulation of SFAs was associated with nZnS treatments. However, detailed studies are required for a mechanistic understanding of the effects of nZnS application on FA production/regulation. Moreover, except for Cu, significant increases in Zn, Fe, and Mn accumulations were found in the roots and leaves of the treated plants, signifying the important role of nZnS in micronutrient accumulations. Overall, our study showed a significant positive role of nZnS in the nutritional status of the mungbean plants. This study contributes to the body of knowledge about plant responses to the nZnS.

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CHAPTER 4

A Comparative Life Cycle Study of effects of nZnS, nZnO and $ZnCl_2$ on Mungbean Plant Yield and *Bradyrhizobium* Symbiosis

4.1. Introduction

Nanoparticles (NPs) are of great interest due to their superior physicochemical properties and potential effects on ecology and human health. Applications of NPs in different sectors including agriculture, food industry, environmental remediation, energy and diagnostics etc., are growing exponentially, raising environmental concern.[1] In agriculture, NPs have primarily been applied as nanofertilizers, nanopesticides or as nanosensors to modulate plant growth and diseases control.[2] In recent years, developments of novel nano-agrochemicals are getting great attention of researchers to support the necessary increase in global food production in a sustainable way.[3]

Zinc (Zn) is an essential micronutrient for plant enzymes and proteins synthesis. Zn is also associated with chlorophyll, carbohydrates and auxins formation in plants. Zn deficiency is one of the most widely distributed micronutrient problems limiting crop production in the world. In soil, Zn can be present in various forms e.g., nZnS, ZnO, $ZnCO_3$, and $Zn_3(PO_4)_2 . 4H_2O$, but it is available to plant only in divalent form (Zn^{2+}). Soil available Zn is deficient when its concentration is <1 mg Zn/kg, sufficient at 1–2 mg Zn/kg, and excessive at >7.5 mg Zn/kg. Therefore, Zn fertilizer recommendation could be done according to the sufficiency and deficiency indices of soil.[4] Traditionally, micronutrients are employed to plants in soluble forms (e.g. $ZnSO_4$, Zn-EDTA), but the efficiency is low.[5] When regular fertilizers are broadcast in the soil, most ions are adsorbed

to the soil colloidal particles, immobilized by microorganisms or leached, only a fraction is used by the plants.[6] However, in alkaline and calcareous soils Zn can undergo rapid transformation with hydroxides and carbonates to form chemical precipitates e.g., Zn $(OH)_2$ and $Zn_2CO_3(OH)_2$, become unavailable to crops. Therefore, an important challenge is how to enhance Zn bioavailability by plants, in safer and affordable way.

In the recent years, numerous papers have reported the potential of NPs to provide a slow-release delivery of micronutrients to plants, but there is still limited work demonstrating this for agriculture. For instance, Bandyopadhyay et. al., (2014), demonstrated a controlled-release polyphosphate micronutrient fertilizer containing Zn, Fe, Mn, and Cu was able to increase rice yield than conventional micronutrient salts.[7] Also, most recent papers reported positive effects of NPs on plant germination, growth and performances. For instances, increased seed germination and seedling growth, and improved photosynthetic efficiency, biomass and total protein, sugar, nitrogen, and micronutrients were observed in various crop plants; e.g., *Glycine max*, *Vigna radiata* and *Cicer arietinum, Solanum lycopersicum*, and *Triticum aestivum*.[8, 8b, 9, 10] In our recent studies also, we have shown that applications of nZnS at low concentrations promoted mungbean growth and nutritional status of the plants.[11 11b] However, effects of Zn NPs on the life cycle of mungbean plants were yet to be done. Although, nZnO is known to have plant growth promoting activities[12], nZnS also possess uniquely improved physiochemical properties that could serve as

novel fertilizers.[11a] For instance, applications of nZnS at 15 ppm concentration in *Brassica juncea* seedlings resulted in improved growth and antioxidant levels in the treated plants.[13] Likewise, in our previous study, mungbean grown hydroponically showed increased shoots-roots length and improved photosynthesis and antioxidant status with nZnS at 0.1, 0.5 and 1 mg L^{-1} treatments.[11a] But the information for yield, food quality and safety in NPs treated crops is limited. Thus the potential application of NPs as supplement or as fertilizer is an attractive prospect that is recently being explored.[14] Therefore, to develop more efficient fertilizers a comparative study among nZnS, nZnO and $ZnCl_2$ is essential.

Studies have demonstrated that both nZnS and nZnO can exhibit negative impacts on microorganisms also.[15, 16] The Rhizobium–legume symbiosis is a naturally occurring phenomenon between soil microbes and legume plants. A healthy Rhizobium–legume interaction lifts plant growth and crop productivity by providing bioavailable nitrogen to the plants. Soil contamination by ZnNPs can threaten agricultural productivity and sustainability by disturbing Rhizobium–legume symbiosis but little is known about the impacts of NPs on the Rhizobium–legume symbiosis.

The aims of this study were (i) to compare the impact of nZnS, nZnO and $ZnCl_2$ on the plant's overall health, yield and quality and (ii) to determine the effects of nZnS, nZnO and $ZnCl_2$ on *Bradyrhizobium* symbiosis to inform the associated environmental risks. In this study, we elucidated nZnS as

nanofertilizers in comparison with that of already recognised Zn source i.e., nZnO and $ZnCl_2$, which could enrich the soil and plant with optimal Zn and improve crop health and productivity in the field environment.

4.2. Materials and Methods

4.2.1. Zinc Sulphide and Zinc Oxide Nanoparticles

nZnS was synthesized by chemical precipitation method at 80° C under nitrogen environment. A detailed synthesis procedure and characterisations of nZnS have been given in our previous study.[11a] Briefly, the sizes of synthesised nZnS were ≤ 20 nm with an average diameter of 13.3 ± 0.3 nm. The hydrodynamic diameter was ≤ 100 nm and zeta potential was – 4.84 mV at 25° C (at pH 7). nZnO dispersion was purchased from Sigma Aldrich (catalogue no.721077). The hydrodynamic diameter of nZnO was < 100 nm with an average particle size ≤ 40 nm measured using an aerodynamic particle sizer (APS) spectrometer. Zeta potential of nZnO was + 42 mV at 25° C measured using Malvern zetasizer. Characterization of this batch of nZnO has been previously published by Wang et. al., (2013).[17]

4.2.2. Experimental Set up

Our experiments followed a three factorial design, which comprised of nZnS, nZnO and ionic Zn ($ZnCl_2$, as positive control), with five different concentrations (0, 0.01, 0.1, 1 and 10 mg kg^{-1} of soil). The whole setup was divided into two groups (SET-1 and SET-2). Both the groups contained a total of 90 pots with two plants each (n=180 seedlings). To avoid any spatial effects, a completely randomized design (CRD) with three replicates per treatment was

followed. Both the groups were inoculated with *Bradyrhizobium*. SET-1 containing 45 pots was used for nodulation study (plants were harvested after 14 days of inoculation) and SET-2 with the remaining 45 pots was harvested after 60 days.

4.2.3. Suspension Preparation

The suspensions of nZnS and nZnO at 0, 0.01, 0.1, 1 and 10 mg L^{-1} were prepared as compound based concentrations in Millipore water and were sonicated at 25° C for 1 h before applying. $ZnCl_2$ solution at 0, 0.01, 0.1, 1 and 10 mg L^{-1} were prepared by dissolving $ZnCl_2$ salt with Millipore water. Freshly, prepared suspensions were used each time.

4.2.4. Seed Preparation for Pot

Mungbean seeds were surface sterilized in 70% ethanol for 30 sec with agitation, followed by rinsing with double distilled water (ddH_2O). Then seeds were immersed in 5% sodium hypochlorite solution for 10 min with agitation and then rinsed 10 times with ddH_2O before experimental treatments. After surface sterilization, seeds were imbibed with different experimental concentrations of nZnS, nZnO and $ZnCl_2$ (0, 0.01, 0.1, 1 and 10 mg L^{-1}) and kept in dark for 4 h. After that, treated seeds (n=70) were kept for germination in the Petridishes on filter paper moist with respective treatment suspensions, for 24 h in dark at 28° C, ddH_2O was used as control. After germination, seeds

germination rate were calculated[11a] and the seeds that developed a primary root of at least 1 mm were used for further study.

4.2.5. Soil Treatments and Pot Preparation

The plants were grown in field soil in the Bidhan Chandra Krishi Viswavidyalaya, Mohanpur campus, West Bengal, India. The soil was air-dried and sieved (2 mm mesh) before use. The soil type was silt loam (see supplementary Table S1 for physicochemical properties of the soil) with a pH of 7.6. The background Zn concentration in the soil was 0.75 mg Zn kg^{-1}. Pots were prepared by amending the soil with the Zn compounds (nZnS, nZnO and $ZnCl_2$) at 0, 0.01, 0.1, 1 and 10 mg kg^{-1} of soil. For amending the soil, each Zn compound was weighed and suspended in 100 mL of ddH_2O to achieve the desired concentrations. Aqueous suspensions of nZnS, nZnO and $ZnCl_2$ were hand mix with 6 kg of soil per pot for 30 min to ensure homogeneity. Three replicates of each treatment were prepared. Untreated soil was served as a negative control.

4.2.6. Bacterial Inoculation

Bradyrhizobium was collected from the Survey, Selection and Mass Production Unit of Bidhan Chandra Krishi Viswavidyalaya (BCKV), Mohanpur-741252, West Bengal, India. The bacterial inoculum (10^6-10^7 CFU mL^{-1}) was prepared by diluting pure culture of *Bradyrhizobium*, using yeast extract manitol (YEM)

broth medium, achieved by dilution based on optical density. Pots (control and Zn amended soil) were then inoculated with *Bradyrhizobium* by hand mixing the bacterial inoculums with the soil thoroughly. Thereafter, two germinated mungbean seeds with uniform sizes were sown carefully in each pot. Seeds were placed about 1 cm deep in the soil and covered with a thin layer of soil. The pots were watered every day. For the foliar treatment, the plant shoots were sprayed once after 30 days before fruiting with 5 ml of treatment solutions.

4.2.7. Zinc Release in Soil

Zn concentration in soil solution was determined for nZnS and nZnO only, following Rawat et. al., (2018),[18] with few modifications. $ZnCl_2$ was not considered in the analysis because of its readily soluble nature in water. For the experiment, three replicates each with 20 g soil were mixed with the NPs (nZnS and nZnO) and 30 ml ddH_2O to make an aqueous suspension of 10 mg kg^{-1} NPs concentration. The aqueous suspensions were then shaken at 250–300 rpm for 24, 48 and 72 h. After shaking, the samples were allowed to settle for 4 h. Thereafter, 15 ml of the supernatants were separated and centrifuged at 5000 rpm for 15 min. 10 ml of the subsequent supernatants were decanted and centrifuged again at 5000 rpm for 30 min. Another round of centrifugation was carried out with 8 ml of the resultant supernatants at 15,000 rpm for 30 min. The process was repeated three times to filter out the particles in the suspensions and contain just the dissolved ions in the suspensions. Finally, 5 ml

of the soil suspensions were analyzed by Atomic Emission Spectroscopy (Agilent Technology 4210, MP-AES, USA) for determining the concentration of dissolved Zn^{2+} in each sample.[18-19]

4.2.8. Plant Harvest and Micronutrient Assessments

At harvest (60 days post-planting), plants were washed in ddH_2O and rinsed three times with 4% HNO_3 and Millipore water. Plant height (aboveground), roots and leaves dry biomass, number of root nodules and fruits agronomical parameters were recorded for each plant. Plants dry biomasses were recorded by drying the samples at 80° C for 24 h. For elemental analysis, collected samples (leaves, roots and fruits) were oven dried at 60° C for 72 h; the dried samples were then ground to a homogenized powder and digested in a microwave-accelerated reaction system using a mixture of plasma pure HNO_3 and H_2O_2 (1:4) then micronutrient contents were quantified using Atomic Emission Spectroscopy (Agilent Technology 4210, MPAES).

4.2.9. Scanning Electron Microscope Analysis of Root Bacteria

Rhizobia could undergo morphological changes in response to environmental stresses, e.g., foreign compounds or pathogens.[20,21] Therefore, to investigate the effect of Zn compounds (nZnS, nZnO and $ZnCl_2$) on bacterial morphology, fully grown nodules were taken and analyzed through scanning electron microscope (SEM). For SEM analysis, the treated and control roots nodules

were collected after 14 days of inoculation. Nodules were cut in thin longitudinal sections (L.S.) and fixed with 2% glutaraldehyde in 100 mM sodium phosphate buffer at 4° C for 2 h, followed by postfixing the samples for 2 h with 1% osmium tetroxide solution. The samples were then dehydrated with graded ethanol. After that, the samples were coated with platinum for 60 s (ca. 1 nm platinum layer) by using a Sputter Coater and then observed under SEM (JEOL JSM-7600F, with Energy Dispersive X-ray, EDX). Released rhizobia (*Bradyrhizobium*) within the nodule cytoplasm were imaged.

4.2.10. Histological Study of Root Nodules

For the histological study, collected nodules were immediately fixed with 4% formaldehyde in 100 mM sodium phosphate buffer (pH 7.2) and stored overnight at 4° C. Then, paraffin wax embedded tissue blocks were prepared and 4–5 µm thin longitudinal sections (L.S) were made using a microtome. Sections were picked up on glass slides (76 mm × 26 mm). Then, the sections were stained with Hematoxylin/Eosin (H/E) stains.[22] Briefly, slides with sections were fixed in alcohol and rinsed with ddH_2O for 30 sec. Then, slides were dipped into Coplin jar containing Mayer's hematoxylin and agitated for 30 sec and rinsed in ddH_2O for 1 min. After that, slides were stained with 1% eosin Y solution for 10–30 sec with agitation followed by dehydrating the slides with two changes of 95% alcohol and two changes of 100% alcohol for 30 sec each. Then, alcohol was extracted with two changes of xylene. Finally, slides were

mounted with cover slips using glycerol and examined under light microscope (LM, Nikon EclipseE600, Japan).

4.2.11. Statistical Analysis

The data were expressed as mean ± standard error of three replicates. Statistical differences among treatments were determined using one-way analysis of variance (ANOVA) followed by Tukey's test at a significance level of 0.05.

4.3. Results and Discussions

4.3.1. Seed Germination

Seed germination was significantly altered by different concentrations of Zn compounds (**Figure 1**). A significant increase in the germination rate was recorded for NPs (nZnS and nZnO) treated seeds, whereas no significant change was found in ionic Zn ($ZnCl_2$) treated seeds at 0.01, 0.1 and 1mg L^{-1} concentrations ($F=150.833$, $p < 0.001$). Seed germination increased by 14, 17 and 10%, respectively in nZnS treated seeds. For the nZnO treatments, germination rates increased, respectively to 14, 15 and 8%. However, compared to the control, significant decreases in the germination rates were observed at 10 mg L^{-1} concentration of Zn compounds. Ionic Zn ($ZnCl_2$) treatment showed maximum inhibition in the seed germination than both the NPs. As $ZnCl_2$ was readily soluble in water, it produced maximum amount of Zn^{2+} ions at a time that could be a reason of its phytotoxicity. Recent studies have shown positive effect of NPs that can promote seed germination.[23] For instance, nZnO treatment promoted seed germination in onion at lower concentration (20 μg ml^{-1}), but at higher concentration (40 μg ml^{-1}) showed reduction in seed germination.[24] Similar to our previous study, nZns treatment also enhanced seed germination at low concentrations (0.1–1 mg L^{-1}).[11a] NPs can create new pores in roots, therefore, positive effect of NPs on seed germination may be related due to increased availability of minerals and retention of water.[25] Despite the

increasing germination rates at lower concentrations, higher concentration of NPs showed inhibitory effect. The phytotoxicity was due to a disruption in the water and nutrient pathways in plants.[26] Our result confirmed that although Zn is an essential element for plant growth, excess Zn might cause retardation in seed germination, result in growth inhibition and can produce toxic symptoms.[27] Thus, our study was consistent with the previous reports and showed positive effect of NPs (nZnS and nZnO) in seed germination of mungbean plant at low concentrations.

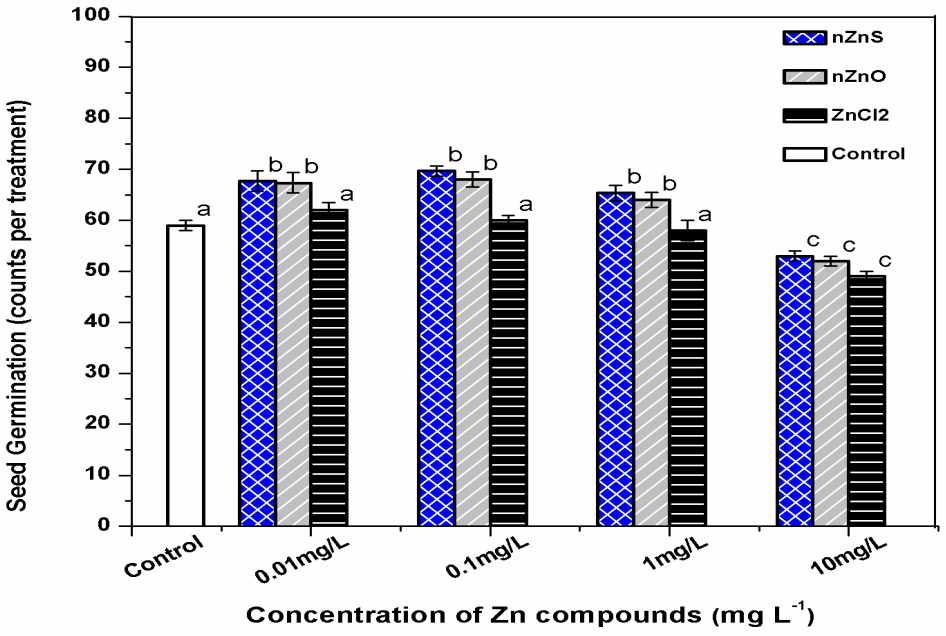

Figure 1. Germination (mean± SE) of mungbean seeds treated with nZnS, nZnO and $ZnCl_2$ at 0, 0.01, 0.1, 1 and 10 mg L^{-1} concentrations. Letters on bars designate significant changes as per one-way ANOVA and the Tukey test ($p \leq 0.05$).

4.3.2. Particles Dissolution Study

The pristine Zn content of soil was deducted from the measured values to obtain the absolute Zn^{2+} ion concentration. Result showed that the concentration of released Zn^{2+} consistently increased over time for both the NPs with higher dissolution recorded for nZnO over nZnS (**Figure 2**). The Zn^{2+} content in the soil solution from nZnO was 12.44, 10.69 and 8.24%, greater than nZnS at 24, 48 and 72 h, respectively ($F= 840.7$, $p< 0.001$). To the best of our knowledge this is the first report to compare Zn^{2+} ion release from two different sources i.e., nZnS and nZnO and their impact on mungbean growth and yield. Also, as there were very few such studies present in the soil and limited knowledge made the comparison difficult. The medium exerts a strong influence on NPs dissolution. However, Zn^{2+} ion release from nZnO in soil was reported by some researchers.[19, 28] For instance, Milani et. al., (2012) reported the adsorption affinity of nZnO was greater than that of readily soluble form of Zn, which suggested nZnO were retained more strongly than soluble Zn in soils.[29] Also, Zn^{2+} ion release from nZnS in Milli-Q water was reported in our previous study, which demonstrated slow and concentration dependent release of Zn^{2+} from nZnS.[11a] These earlier reports in some ways supported the results found in the current study. Also, nZnO are known for their higher dissolution rate[19], bond in ZnO is more ionic than that in ZnS.[30] While nZnS has slight agglomerative property[11a] could reduce the diffusion rate of Zn^{2+} from nZnS. This might be the reason for observing the difference in the Zn dissolvability between the two NPs

(nZnS and nZnO). Overall, no prior data were available to explain this finding; however the higher dissolution property of nZnO than nZnS cannot be discounted needed further study. However, NPs with slow release capabilities could potentially lower the amount of micronutrient lost due to leaching from soils, and increased availability to plants but detailed studies are lacking.[31]

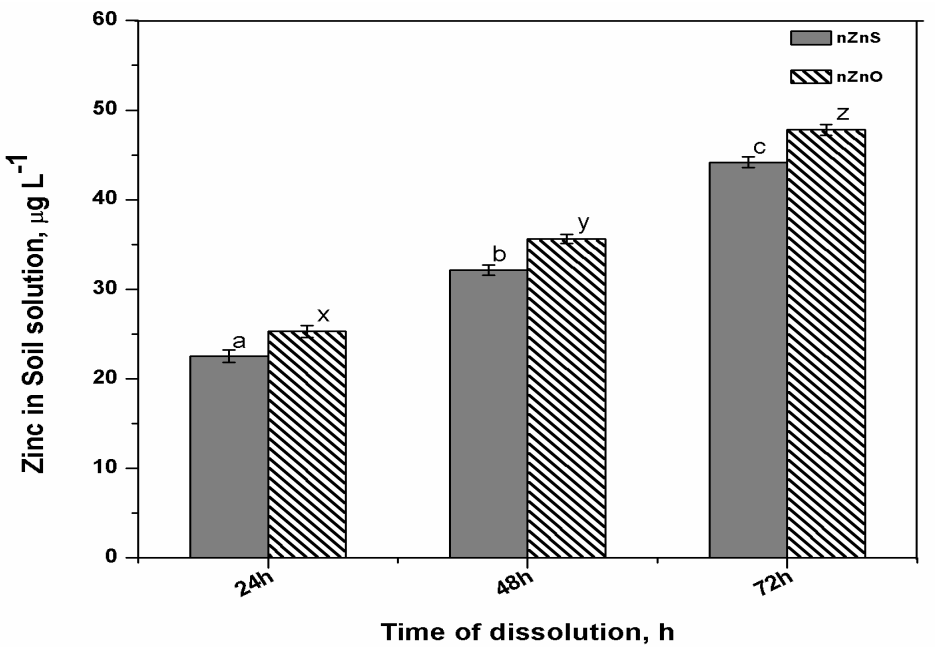

Figure 2. Relative dissolution (mean ± SE) of nZnS and nZnO in soil solution (in ddH_2O), deducting the pristine zinc present in the soil. Letters on bars designate significant changes as per one-way ANOVA and the Tukey test ($p \leq 0.05$).

4.3.3. Effects of Zn Compounds on Plant Height, Dry Biomass and Number of root nodules/plant

At maturity, plant height, dry biomass and root nodules per plant were quantified as indicators of plant health, presented in **Figure 3** (A–D). Results revealed that the average height (aboveground) of the NPs (nZnS and nZnO) treated plants was significantly increased in low concentration, as compared to the control plants (**Figure 3A**). The maximum height promoted was 34%, found in the plant treated with 0.1 mg kg^{-1} nZnS ($F=182.392$, $p<0.001$). It was noticed that in case of nZnO and $ZnCl_2$ treated plants, reduction in plant heights occurred from 1 mg kg^{-1} concentration. On the other hand, 10 mg kg^{-1} treatments imposed a higher reduction in plant height in all the Zn compounds (nZnS, nZnO and $ZnCl_2$). Maximum reduction by 67% was found in plant exposed to 10 mg kg^{-1} concentration of $ZnCl_2$. In case of plant dry biomass, leaves and roots dry biomasses were significantly increased in nZnS and nZnO treated plants at 0.01 and 0.1 mg kg^{-1} concentrations (**Figure 3B–3C**), compared to the control. The highest increases in leaves and roots dry masses were found in 0.1 mg kg^{-1} concentration of nZnS. At 0.1 mg kg^{-1} nZnS treatment, mungbean plant had more than twice leaves dry biomass than the control ($F=38.673$, $p<0.001$). However, at 10 mg kg^{-1} concentration, leaves and roots dry biomasses were significantly decreased in all the Zn compounds. The highest reduction was found in leaves dry biomass at 10 mg kg^{-1} concentration of $ZnCl_2$. In this study, average number of root nodule per plant remained statistically unaltered

at 0.01, 0.1 and 1 mg kg^{-1} of nZnS while, a 62% reduction recorded in 10 mg kg^{-1} nZnS treatment (**Figure 4D**). Similarly, no significant differences were also found at 0.01 and 0.1 mg kg^{-1} of nZnO and $ZnCl_2$ treatment concentrations. But at 1 and 10 mg kg^{-1} nZnO and $ZnCl_2$, root nodules per plant were significantly reduced ($F=21.123$, $p<0.001$). Root morphology was shown in supplementary information (SI), **Figure S1**.

Previous studies, have documented the similar results in mungbean and other plants. For example, Raliya et. al., (2015), reported increase in tomato plant height up to 250 mg kg^{-1} nZnO concentration.[32] Thapa et. al., (2019), demonstrated nZnS treatments at 0.1–1 mg L^{-1} significantly increased roots–shoots weights of mungbean plant after 10 days of growth.[11a] Rossi et. al., (2019), reported nZnO positively affected the fresh and dry weights of roots and leaves of the coffee plant compared to the control and $ZnSO_4$ application.[33] According to Kah et. al., (2018), NPs can enhance plant growth and nutritional quality by 20–30%, compared to the conventional products.[3] However the mechanism behind the plant biomass increment by NPs has yet to be determined.[32] Many researchers are working on this to emphasize the beneficial role of NPs in plants. In this line, Larue et. al., (2012), explained that the higher surface reactivity of NPs could enlarge the pores or create new ones that may elevate nutrient uptake by plant roots and subsequently increase plant growth.[34] The increased biomass could be correlated with the improved chlorophyll content and photosynthesis because Zn is an essential micronutrient for plants to

execute many physiological activities including biosynthesis of proteins and enzymes, chlorophyll, and normal functioning of the metabolic processes.[35] Therefore, it is possible that the treatments of Zn compounds might have altered biochemical processes inside the mungbean plants and potentially promoting or inhibiting plant growth at different concentrations. In our study, nZnO and $ZnCl_2$ were found to be more phytotoxic than nZnS to mungbean plants in terms of reduction of plant height, dry biomass and root nodules per plant, which needs further detail study. However, toxicity was much higher in ionic Zn treatments ($ZnCl_2$) compared to the NPs treatments. Zn amended as $ZnCl_2$ affected plant behaviour to a higher extent than Zn applied as nZnS or nZnO. On the other hand, plants were healthy in lower concentrations. The amount of Zn released from NPs and accumulated inside the plants was crucial for plant growth hence, at low concentrations, it acted as a growth promoting factor for mungbean plants. Our results were in accordance with Raliya et. al., (2015), who reported that there was a critical concentration of NPs up to which the plant's growth and development were promoted but beyond that no improvement occurs.[32] Similarly, Mahajan et. al., (2011), reported that nZnO at 20 ppm concentration increased roots–shoots biomass of mungbean seedlings grown in agar medium, but at the highest concentration of 2000 ppm roots–shoots biomass was decreased.[8b] As previously discussed, Zn in moderate amounts is beneficial for all organisms including plants but, when present in excess, can be phytotoxic. Zn induced toxicity is associated with an inhibition

of growth and interference in several metabolic processes, and induction of oxidative stress, compromising the homeostasis/redox state in plant.[36] Therefore, our findings suggest that applications of Zn compounds at 10 mg kg^{-1} concentration are toxic for plant growth whereas; at low concentration NPs can enhance plant growth and biomass. Also, in this study, nZnS acted as a better growth–enhancing factor while, $ZnCl_2$ showed negative effects to mungbean plants.

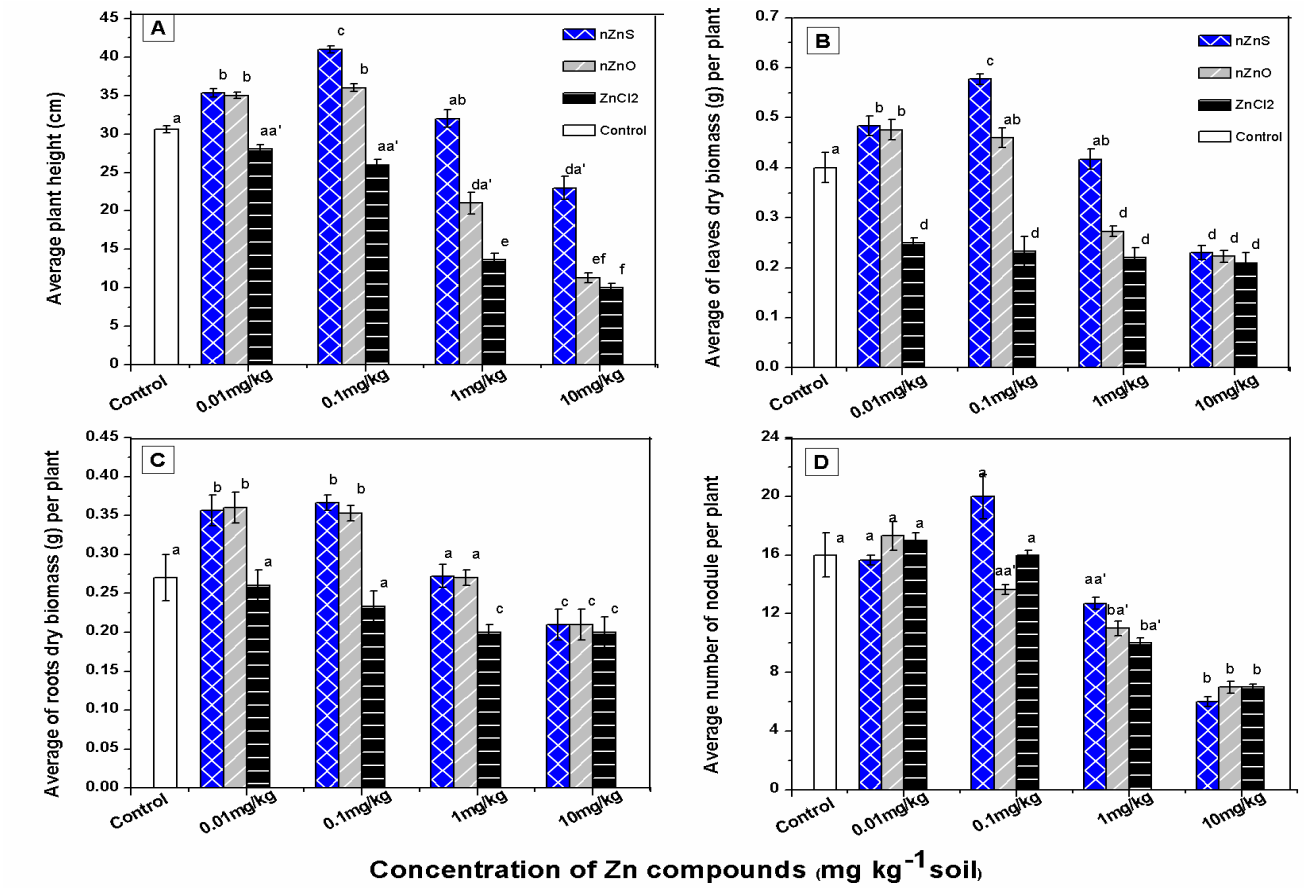

Figure 3. (A) Plant height, (B) leaves dry biomass, (C) roots dry biomass and (D) number of nodules per plant of mungbean plants cultivated for 60 days in soil amended with nZnS, nZnO, and $ZnCl_2$ at 0 (control), 0.01, 0.1, 1 and 10 mg kg^{-1} soil. Data are averages of three replicates ± SE. Different letters designate significant change as per one-way ANOVA and the Tukey test ($p \leq 0.05$).

4.3.4. Effects of Zn Compounds on Fruit Agronomical Parameters

The effects of applications of Zn compounds (nZnS, nZnO and $ZnCl_2$) on the fruit (pods) agronomical parameters were presented in **Figure 4 (A–E)**. As evident, plants treated with NPs (nZnS and nZnO), produced more pods than the control and ionic treatment ($ZnCl_2$, **Figure 4A**). The maximum increase of 58% was found at 0.1 mg kg^{-1} nZnS treated plants, over control. However, all the Zn compounds at 10 mg kg^{-1} concentration significantly decreased the average number of fruits per plant by 56% in nZnS, 58% in nZnO and 66% in $ZnCl_2$ ($F= 76.025$, $p<0.001$). Previously, 250 mg kg^{-1} of $nTiO_2$ significantly increased fruit biomass in tomato plant by 70%, compared to the control.[32] Similarly, foliar spray of nZnO on tomato at 50 ppm increased number of fruits per plant.[12] In addition, Elmer and White et. al., (2016), also reported that foliar treatment of nCuO significantly increased tomato biomass and yield in both greenhouse and field experiments.[37]

In our study, nZnS treatment did not alter the average length of pods per plant up to 1 mg kg^{-1} concentration (**Figure 4B**). At 10 mg kg^{-1}nZnS, the average length of pods per plant was reduced by 45%, compared to the control. Whereas, nZnO treatments reduced the average length of pods per plant by 45 and 60% at 1 and 10 mg kg^{-1} concentrations, respectively. Also, $ZnCl_2$ reduced the average length of pods per plant by 56 and 67% at 1 and 10 mg kg^{-1}, respectively, compared to the control ($F=19.946$, $p<0.001$). Morphology of control and treated pods were shown in SI, **Figure S2**. Similarly, none of the

nZnS treatments significantly affected average number of seeds per pod, (**Figure 4C**). However, the NPs treated seeds were relatively bigger and heavier than the control seeds (SI, **Figure S2**). nZnO treatment at 10 mg kg^{-1} decreased the average number of seeds per pod by 52% and $ZnCl_2$ at 1 and 10 mg kg^{-1} decreased average number of seeds per pod by 51 and 59% respectively, compared to the control ($F=31.758$, $p<0.001$). On the other hand, for NP treated plants, significant increases in the average weights of the pods per plant was occurred, (**Figure 4D**). The maximum increase of 71% was recorded at 0.1 mg kg^{-1} nZnS treatment, over control. At 10 mg kg^{-1}nZnS, average weight of pods per plant reduced by 31%. Similarly, nZnO at 1 and 10 mg kg^{-1} reduced average weight of pods per plant by 40 and 50% respectively, compared to the control. Whereas, in case of $ZnCl_2$, the average weight of pods per plant reduced at all the treatment concentrations by 35, 40, 45 and 64% respectively ($F= 25.565$, $p<0.001$). The effects of Zn compounds on average seed yield were presented in **Figure 4E**. The highest yield was recorded at 0.1 mg kg^{-1} nZnS. Whereas, 45 and 50% decreases in yield were recorded in nZnS and nZnO treatments respectively, at 10 mg kg^{-1} concentrations. In case of $ZnCl_2$, seed yield decreased by 50% at 1 and 10 mg kg^{-1} concentrations ($F = 32.557$, $p < 0.001$).

Previously, Rico et. al., (2014) and Kole et. al., (2013), found that application of nCeO and carbon NPs increased wheat and bitter melon yield by 36.6 and 128%, respectively.[38,38b] Lopez et. al., (2019), reported that nZnO application in habanero peppers (*Capsicum chinense*) at 1000 mg L^{-1} improved fruit yield and

nutritional quality.[39] Also, Hernandez et. al., (2019), reported that Selenium (Se) NPs and copper (Cu) NPs at 20 and 10 mg L^{-1} concentrations increased the average tomato fruit weight by 25% compared to the control.[40] Conversely, Wang et. al., (2012), reported no significant impact of periodic exposure of $nCeO_2$ on the size and average weight of tomato fruit.[41] A comprehensive review article by Rico et. al., (2011), listed several NPs as having positive, non-consequential, or negative effects on different food crops.[42] Our results were consistent with the previous reports and confirmed the increase yield of mungbean by the NPs treatments at low concentrations while, $ZnCl_2$ had clearly negatively impacted plant health and productivity.

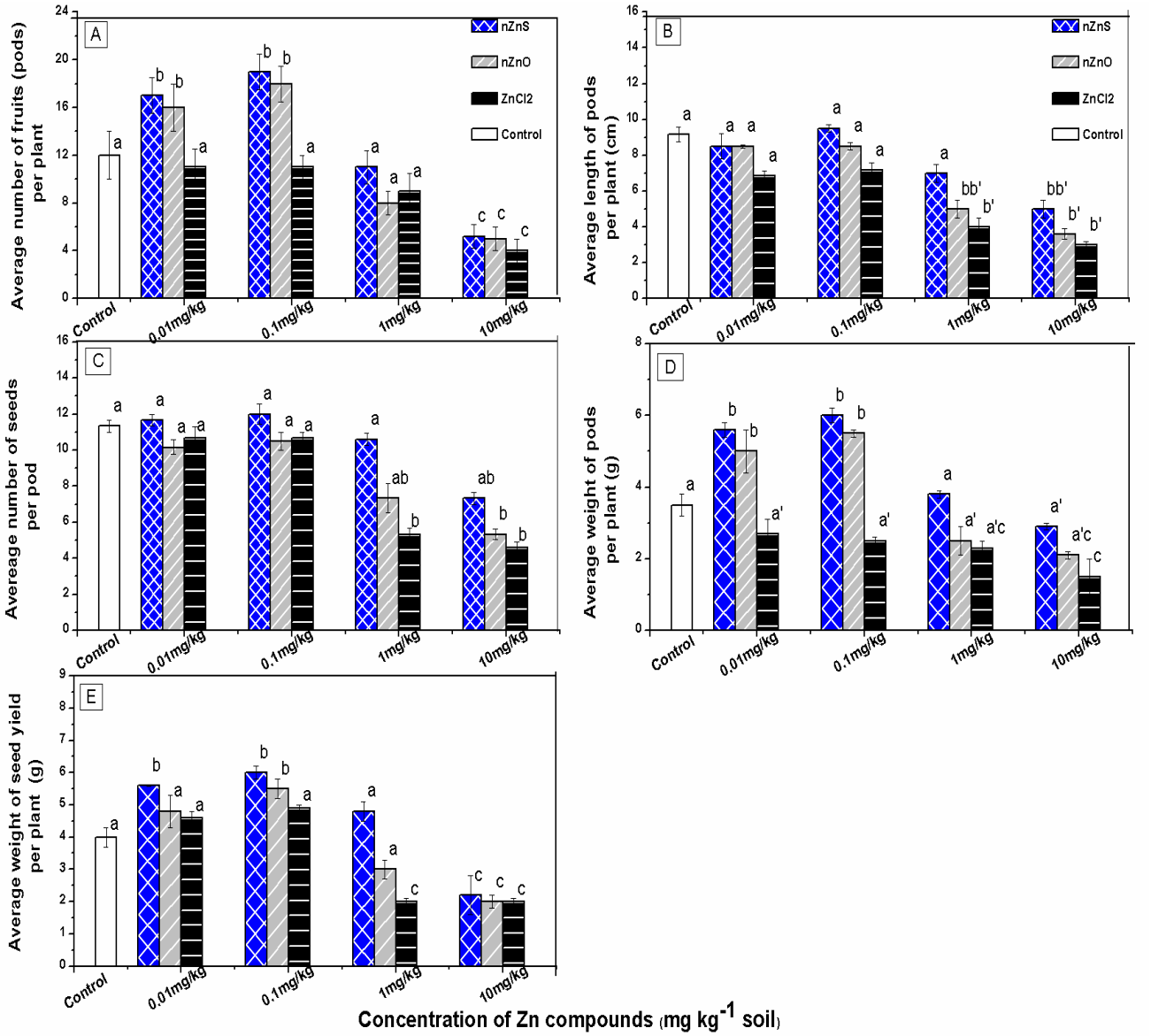

Figure 4. (A) Average number of fruits (pods) per plant, (B) average length of pods per plant, (C) average number of seeds per pod and (D) average weight of pods per plant and (E) average weight of seed yield per plant of mungbean plants cultivated for 60 days in soil amended with nZnS, nZnO, and $ZnCl_2$ at 0 (control), 0.01, 0.1, 1 and 10 mg kg^{-1} soil. Data are averages of three replicates ± SE. Different letters designate significant change as per one-way ANOVA and the Tukey test ($p \leq 0.05$).

4.3.5. Effects of Zn Compounds on micronutrient contents of Fruits

Effects of Zn compounds (nZnS, nZnO and $ZnCl_2$) on micronutrients (Zn, Cu, Fe, and Mn) content of 60 days old mungbean fruits were shown in Table 1. Compared to control, Zn concentration in the fruits of all the treated plants increased significantly ($F= 10.508$, $p<0.001$), with nZnS and nZnO treatments showed maximum increase. In our study, Zn level increased along with the increased concentrations of Zn compounds. The highest accumulation was observed at 10 mg kg^{-1} nZnO treatment with an average of 4 mg kg^{-1} of Zn in fruit i.e., 173% increase over the control. Previously, dose-dependent increase in metal accumulation was found in tomato fruit treated with CeO_2 NPs (0.1–10 mg L^{-1}).[41] Since, the distribution of NPs in plants, including their edible parts, is a food safety concern; accumulation of Zn in higher levels could generate phytotoxicity at high NP treatment concentrations. According to the Food and Nutrition Board, Institute of Medicine (Washington DC), the recommended dietary intake of Zn for adults was 8–11mg per day.[43] Thus, our data could indicate that Zn content in fruits was in threshold level even at higher treatment concentration therefore, phytoxicity of nZnS and nZnO depend not only on metal or ion accumulation in plants but on the overall plant health.[19, 44] In this study, the NPs treated plants accumulated more Zn in fruits than the $ZnCl_2$ treated plants. This result indicated that NPs induced more Zn uptake and translocation in the fruits than the ionic source of Zn ($ZnCl_2$), which followed

the previous report by Yang et. al., (2021), which showed that ZnO NPs enhanced Zn concentration of brown rice by 13.5 to 39.4%, compared to the conventional fertilizer, $ZnSO_4$.[45] Moreover, it has been demonstrated by many researchers that Zn fertilization improved production and quality of fruits by positively impacting β carotene and lycopene contents in tomato[46], capsaicin content in habanero pepper[39], caffeine in coffee[47] etc. Our finding in this study was consistent with the previous studies.

No significant alterations were found in Cu and Mn accumulations in fruits. Whereas, significant increases in Fe content was recorded at 0.01 mg kg^{-1}nZns and nZnO, 0.1 mg kg^{-1} $ZnCl_2$ and 10 mg kg^{-1} nZnO treated plants ($F=12.352$, $p<0.001$). A synergistic effect of Zn and Fe has been found in our previous study also, where nZnS application increased Fe content in mungbean plant.[11b] Our finding was in line with Rengel et. al., (1998), where deficiency in Zn led to the deficiency in Fe content, due to prevention of transfer of Fe from root to shoot in wheat under Zn deficient condition.[48] Also, there were many factors that affected metal uptake in plants such as soil pH, microorganisms, metal immobilization in the root cell walls and chelation with organic and inorganic matters in the soil.[49] Zn bioavailability for plant decreases by high soil pH, low geogenic Zn levels and high contents of phosphates, clay, natural organic matter, and carbonates [50], therefore, using ionic Zn as fertilizers would not give a beneficial result under these conditions. In our study, the soil was Zn

deficient. Thus, increase in Zn uptake by treated plants indicated the potential role of nZnS and nZnO as a Zn fertilizer. Therefore, our result suggested that nZnS and nZnO had better performance in the uptake and bioavailability of Zn in the plant than ionic $ZnCl_2$. This might be attributed to the slow release of Zn^{2+} from NPs, providing a long term source of Zn.

SL. NO.	Treatments	Zinc (Zn) mg kg^{-1} dry weight	Copper (Cu) mg kg^{-1} dry weight	Iron (Fe) mg kg^{-1} dry weight	Manganese (Mn) mg kg^{-1} dry weight
1.	Control	1.46±0.03 a	1.33±0.01 a	2.1±0.12 a	1.66±0.06 a
2.	0.01mg kg^{-1} nZnS	3.4±0.1 c	1.2±0.02 a	3±0.03 b	1.8±0.00 a
3.	0.01mg kg^{-1} nZnO	3.46±0.22 c	1±0.10 a	3.2±0.15 b	1.8±0.00 a
4.	0.01mg kg^{-1} $ZnCl_2$	2.3±0.3 b	1.3±0.02 a	2.7±0.1 a	1.4±0.00 a
5.	0.1mg kg^{-1} nZnS	3.88±0.02 c	1.2±0.01 a	2.3±0.15 a	1.8±0.01 a
6.	0.1mg kg^{-1} nZnO	3.6±0.3 c	1±0.00 a	2.7±0.006 a	2.8±0.01 b
7.	0.1mg kg^{-1} $ZnCl_2$	2.14±0.1 b	1.33±0.10 a	3.7±0.1 b	1.8±0.01 a
8.	1mg kg^{-1} nZnS	3.74±0.11 c	1±0.002 a	2.56±0.12 a	1.6±0.00 a
9.	1mg kg^{-1} nZnO	3.8±0.4 c	1.2±0.01 a	2.4±0.233 a	1.8±0.03 a
10.	1mg kg^{-1} $ZnCl_2$	2.4±0.5 b	1±0.33 a	2.5±0.03 a	1.6±0.01 a
11.	10mg kg^{-1} nZnS	3.86±0.13 c	1±0.02 a	2.36±0.18 a	1.8±0.00 a
12.	10mg kg^{-1} nZnO	4±0.02 c	1±0.02 a	3.3±0.08 b	1.8±0.02 a

13.	10mg kg^{-1} $ZnCl_2$	2.4±0.1b	1±0.03 a	2.66±0.12 a	1.66±0.03 a

Table 1: Effects of Zn compounds on micronutrient contents of fruits of mungbean plants cultivated for 60 days in soil amended with nZnS, nZnO, and $ZnCl_2$ at 0 (control), 0.01, 0.1, 1 and 10 mg kg^{-1} soil.Data are averages of three replicates ± SE. Letter changes along the column depict significant difference. Same letter are not significantly different at Tukey's test (at p <0.05).

4.3.6. Effects of Zn Compounds on micronutrient contents of Leaves and Roots

Effects of Zn compounds on micronutrient contents of mungbean plant tissues (leaves and roots) were presented in Table 2. Zn contents in the leaves and the roots of treated plant increased significantly. Zn accumulation in plants was in concentration dependant manner. The increase in Zn, varied from 52 to 108% in leaves and 26 to 50% in roots at 0.01–10 mg kg^{-1} nZnS. The increase in Zn, varied from 66 to 120% in leaves and 26 to 57% in roots at 0.01–10 mg kg^{-1} nZnO. In $ZnCl_2$ treated plants, the increase in Zn varied from 80 to 146% in leaves and 35 to 76% in roots at 0.01–10 mg kg^{-1}.The maximum Zn accumulation occurred in leaves because of the foliar spray. Raliya et. al., (2015), reported increased Zn accumulation in the tomato leaves when applied as foliar sprays.[32] In this study, the highest Zn accumulation was found in $ZnCl_2$ treated plants (both in leaves and roots); this was because of the readily soluble

nature of ionic Zn ($ZnCl_2$). For plant growth and development, nutrient demand is not constant along its life cycle[51], it dramatically increases during certain phases like blooming and grain filling.[52] Therefore, Zn NPs could be used for a slow and continuous release of Zn, which could serve as a sustained Zn pool to provide Zn nutrition throughout the life cycle of the plants.[10, 53] Again, application of Zn NPs was found to enhance the amount of Zn in plants like aromatic rice, cowpea, habanero peppers etc.[17, 39, 54] Since, Zn is an essential microelement in plants and it is required for macromolecule synthesis and serves as a regulatory cofactor in protein synthesis. Therefore, the presence of an optimum amount of Zn is required for plant metabolism, yet deficiency is prevalent in part due to the plant's inefficiency in absorbing and translocating the micronutrient.[55] Typically higher than 400 mg kg^{-1} of Zn in dry mass of plant tissue, is toxic to plants.[23, 56] As shown in Tables (1 and 2), none of the treated plants exceeded the threshold level; this result confirmed that Zn^{2+} was not the sole cause of NPs toxicity, rather, the phytotoxicity of NPs at high concentration might be the combined interference of physical and chemical stresses.[44]

Cu level did not vary in roots, while, significant reductions of Cu level in leaves was recorded at 10 mg kg^{-1} concentration of Zn compounds. Higher concentrations of Cu in the soil solution, relative to zinc, can reduce the availability of zinc to a plant (and vice versa) due to competition for the same

sites for absorption into the plant root.[11b, 57] Hence, reduction in Cu content occurred at high concentrations of Zn application. Conversely, significant improvement in Fe contents of leaves and roots of treated plants were recorded. No significant alteration in leaf Mn level was found whereas, roots Mn level decreased in the treated plants. Previously, Imtiaz et. al., (2003), reported that higher Zn rates reduced Mn concentrations significantly in wheat.[58] Our result confirmed that Zn treatments significantly increased Zn level in plant tissues but had minimal effects on Cu, Fe and Mn contents of mungbean plants. This can give further research insight into nutrient enhancement or biofortification in plants to improve the nutritional values of crops.

SL. NO.	Treatments	Zinc (Zn) mg kg^{-1} dry weight		Copper (Cu) mg kg^{-1} dry weight		Iron (Fe) mg kg^{-1} dry weight		Manganese (Mn) mg kg^{-1} dry weight	
		Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
1.	Control	5±0.16 a	14±0.3 a	1.7±0.01 a	2.4±0.02a	1.4±0.01 a	2.2±0.2 a	2.4±0.1 a	1±0.02 a
2.	0.01mg kg^{-1} nZnS	7.6±0.3 b	17.66±0.3 b	1±0.1 a	2.1±1a	1.14±0.02 a	4±0.04 b	1.8±0.03 a	1.3±0.02 a
3.	0.01mg kg^{-1} nZnO	8.33±0.3 b	17.66±0.33 b	1±0.2 a	2±0.02a	1.33±0.06 a	2.47±0.23 a	1.9±0.03 a	1.2±0.01 a
4.	0.01mg kg^{-1} $ZnCl_2$	9±0.5 b	19±0.5bc	1±0.3 a	2±0.02a	2.4±0.04 b	2.99±0.01 ab	2.2±0.2 a	1.1±0.05 a
5.	0.1mg kg^{-1} nZnS	8.6±0.3 b	18.33±0.5 b	1.5±0.03 a	2±0.02a	2.36±0.18 b	2.55±0.27 a	2.2±0.3 a	1.3±0.1 a
6.	0.1mg kg^{-1} nZnO	8.5±0.2 b	20.33±0.3c	2±0.03 a	1.9±0.03 a	2.16±0.16 b	3.3±0.16 b	1.8±0.01 a	1.4±0.02 a
7.	0.1mg kg^{-1} $ZnCl_2$	10.83±0.16 c	19.33±0.8b	1±0.02 a	2±0.2a	2.7±0.1 b	3.9±0.03 b	2.2±0.02 a	1±0.2 a

8.	1mg kg^{-1} nZnS	9.83±0.4bc	19±1bc	1.5±0.2 a	2±0.01a	1.6±0.15 a	2.5±0.2 a	2±0.1 a	1.2±0.3 a
9.	1mg kg^{-1} nZnO	10.5±0.28 c	21.33±0.3 c'	1.8±0.03 a	2±0.02a	0.99±0.01 a	2.15±0.08 a	1.8±0.3 a	0.09±0.00 b
10.	1mg kg^{-1} $ZnCl_2$	12.33±0.3 c'	22.33±0.6 c'	1±0.00 a	1.8±0.3a	1.28±0.3 a	2.16±0.16 a	1.7±0.2 a	0.05±0.00 b
11.	10mg kg^{-1} nZnS	10.43±0.2 c	21±1 c'	0.8±0.00 b	2±0.1a	1.6±0.15 a	2.4±0.2 a	1.8±0.3 a	1.2±0.01 a
12.	10mg kg^{-1} nZnO	11±0.5 cc'	22±0.5 c'	0.7±0.01 b	1.8±0.02a	1.87±0.13 a	2.4±0.2 a	1.8±0.4 a	1.1±0.01 a
13.	10mg kg^{-1} $ZnCl_2$	12.33±0.3 c'	24.66±0.33 d	0.6±0.01 b	2±0.03a	1±0.09 a	2.5±0.16a	2.2±0.2 a	1±0.00a

Table 2: Effects of Zn compounds on micronutrient contents of leaves and roots of mungbean plants cultivated for 60 days in soil amended with nZnS, nZnO, and $ZnCl_2$ at 0 (control), 0.01, 0.1, 1 and 10 mg kg^{-1} soil.Data are averages of three replicates ± SE. Letter changes along the column depict significant difference. Same letter are not significantly different at Tukey's test (at p <0.05).

4.3.7. Translocation

In our study, maximum translocation of Zn^{2+} from leaf to fruit occurred in NPs treated plants while; Zn^{2+} uptake by roots and leaves was higher in $ZnCl_2$ treated plants (**SI, Figure S3**). This was because of the high solubility of $ZnCl_2$ in soil as well as quick uptake in leaf after foliar spray. The translocation factor (TF) was higher in case of NPs (nZnS and nZnO) treatments than $ZnCl_2$ treatment (**SI, TF; Table S2**). The translocation factor (**SI, TF; Table S2**) calculation showed that more Zn^{2+} was translocated to mungbean fruits at 0.01 and 0.1 mg

kg^{-1} of nZnS (TF: 0.44 ± 0.03 and 0.45±0.03) and nZnO (0.41±0.02 and 0.42±0.2) treatments, respectively than from $ZnCl_2$ treatments (TF: 0.25 ± 0.01 and 0.19 ± 0.02). Higher TF signified more translocation of Zn^{2+} and vice versa.[59] There was no significant change in TF of Zn^{2+} from leaf to fruit found in case of $ZnCl_2$ treated plants, possibly Zn^{2+} complexes with organic acids and Zn was sequestered in vacuoles and become less available for transportation.[60] On the other hand, free smaller size NPs were available for transport in the xylem and phloem; hence, higher translocation was observed. In this study, TF of nZnS was higher than the nZnO, probably due to its smaller size and negative zeta potential, compared to the nZnO (positive zeta potential). The silt loam soil predominantly had a negative charge as did the nZnS particles. The apparent repulsion between the two negative charges probably fueled the translocation of nZnS.[61] Moreover, most of the Zn forms are absorbed through the cuticle and stomates. The apoplast is dominated by a negative charge, which is caused by free carboxyl groups of galacturonic acids (galacturonic acids are part of the mid-lamellae pectins and primary cell walls) which, in turn, causes the binding and subsequent accumulation of cations in the apoplast, and their translocation into other organs of the plant difficult.[62] Furthermore, the concentration of Zn in mungbean plant tissues followed the sequence leaves > roots > fruits. Our result was consistent with the Raliya et. al., (2015), where an increase in metal ion accumulation was observed in the leaves with an increase in both TiO_2 and ZnO NPs exposure concentrations.[32] Also, time of Zn application is important factor

determining the effectiveness of Zn application in enhancing Zn concentration.[63] Application of Zn at the late stage produced a higher Zn content of grains than Zn application at early stage.[63] Our result was in correspondence with the previous reports, that foliar application of NPs could significantly increased Zn uptake in leaves and translocated it. However, more elaborated studies are required in order to explain the higher bioaccumulation of Zn^{2+} by NPs and related mechanisms in leaves, compared to ionic treatments.

4.3.8. Effect of Zn compounds on Bacterial Morphology

SEM images (**Figure 5, A–G**), of the nodules of the control plants showed rod shaped bacteria with uniform cellular surface texture. At 0.1 mg kg^{-1} of Zn compounds, bacterial morphology remained unchanged. While, after treating with 10 mg kg^{-1}NPs (ZnS and nZnO), irregularity, swelling or Y-branching (characteristic branching of bacteroid of N_2 fixing bacteria) in bacterial shapes were recorded. At 10 mg kg^{-1} $ZnCl_2$ treatment, the outer membrane of the bacterial cells appeared damaged and wrinkled. Also, a great number of bacteria were either damaged or completely disintegrated found in 10 mg kg^{-1} $ZnCl_2$ treatment. Also, EDX analysis (**SI, Figure S4–S7**) revealed the presence of Zn^{2+} in bacteria, which confirmed the bacterial uptake of Zn^{2+} from Zn compounds. Weight percentages of elements were given in SI (**Table S3–S6**). These SEM results indicated that soil amended with Zn compounds could be uptake by symbiotic bacteria, present inside the root nodules and subsequently damaged

the outer membrane of the bacterial cell wall. Here, the phytotoxicity of NPs at high concentrations could be due to the generation of a high level of free radicals from the NPs. But, the damage was more severe in the case of $ZnCl_2$, because it was fully ionized in the soil and thus plants uptake more Zn^{2+} and bio-accumulate it in the root tissues. Previously, Panwichian et. al., (2011), reported that excess Zn^{2+} altered the cellular morphology of *Rhodobium marinum* (NW 16) and *Rhodobacter sphaeroides* (KMS24) when grown in 0.89 mM (about 55 mg L^{-1}) of Zn^{2+}; the bacteria cells elongated, transformed to filaments (NW 16) or morphed dumbbell shape (KMS24).[64] Again, electrostatic force could favour the attachment of NPs onto bacterial surface suggested that the antibacterial mechanism of NPs was most likely due to direct interactions between NPs and bacterial cell surfaces, which affected membrane permeability. Furthermore, NPs could produce hydroxyl radical, which would inactivate cell growth and exhibit strong antibacterial activity.[16] Thus, our finding was in agreement with the previous reports that Zn is an essential trace element that can form complexes with many enzymes and DNA-binding proteins; therefore, low Zn concentration can accelerate bacterial growth while high concentration of Zn can damage the bacterial cell.[16, 64]

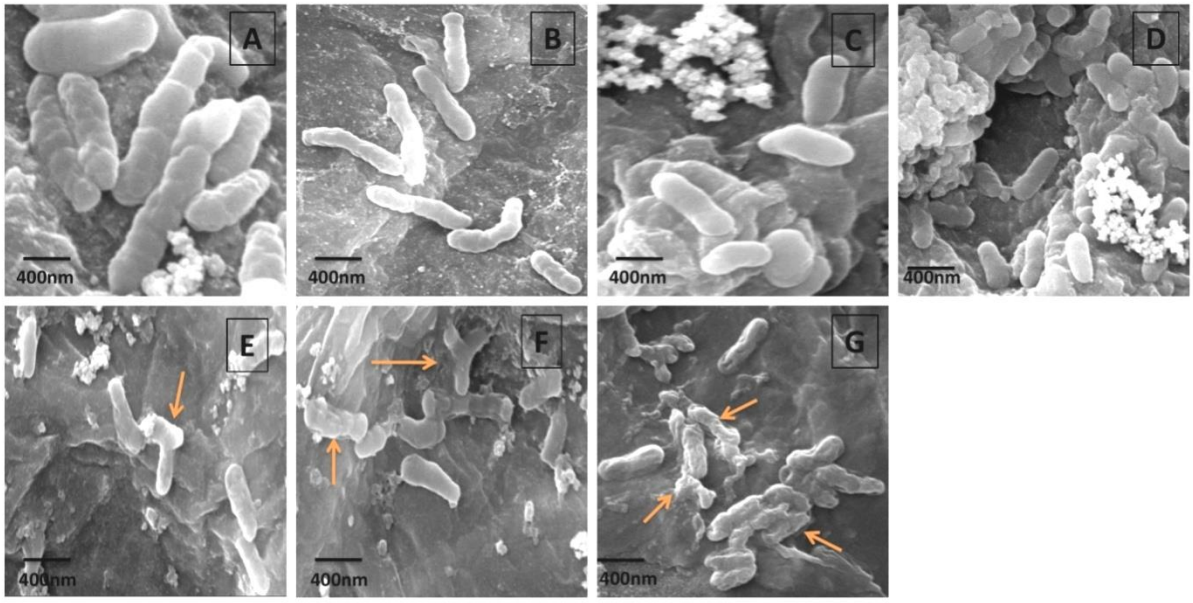

Figure 5. Surface structure of *Bradyrhizobium* on SEM imaging (A) control, (B) 0.1 mg kg^{-1}nZnS, (C)) 0.1 mg kg^{-1}nZnO (D)) 0.1 mg kg^{-1} $ZnCl_2$, (E) 10 mg kg^{-1}nZnS, (F) 10 mg kg^{-1}nZnO and (G) 10 mg kg^{-1} $ZnCl_2$.

4.3.9. Effect of Zn compounds on Ultra-structure of Root Nodule

The effect of Zn compounds (nZnS, nZnO and $ZnCl_2$) on nodule formation and subsequent nodule development was evaluated, shown in **Figure 6 (A–J)**. The light microscopy images showed that control and treated nodules primordia, were similar in structure to the previous report with *V. radiata*[65], i.e., had no permanent meristem and adopted a spherical or globular shape. The mature nodules contained a central zone consisted of both infected and uninfected cells, and a vascular system connecting the nodule to the root, which was surrounded by an inner cortex containing the putative components of an oxygen diffusion

barrier (including glycoprotein-occluded intercellular spaces), and an outer cortex with cells containing calcium oxalate crystals.[66] At lowest concentration (0.1 mg kg^{-1}) of nZnS and nZnO, no alteration in the nodules morphology were noticed, **Figure 6 (A–C)**, which was densely packed with infected cells, (**SI, Figure S8, A–B**). Some infected cells of control and nodules treated with a low concentration of NPs were at the early stage of infection. Most Rhizobia were just being released from the infection droplets and were being differentiated into mature bacteroids, (**SI, Figure S9, A–B**). Whereas, nodule exposed to 1 mg kg^{-1} concentration of NPs, showed relatively lower density of infected cells as compared to the control. Some cells showed early senescence and contained abnormally degraded bacteroids, (**Figure 6, E–F**). For the nodules exposed to 10 mg kg^{-1} concentration of NPs, showed an impaired outer cortex (**Figure 6, H-I**), had low density of differentiating bacteroids (**SI, Figure S8, C**), and a large area of senescence zones (**SI, Figure S9, C**). Our result was in line with Dupont et. al., (2012), confirmed that the NPs attached on the surface of nodules induced stress by generating hydroxyl ions which affected the structure and led to stress-induced senescence in root nodule.[67] Stress induced senescence is a much faster process than normal developmental senescence.[68] In contrast, $ZnCl_2$ treatments showed more drastic effect on nodule formation. $ZnCl_2$ treatments had altered the nodules shape at all the concentrations. While, at 10 mg kg^{-1} $ZnCl_2$ concentration drastically degraded cells were noticed, (**Figure 6, D, G & J and SI, Figure S9, D**). Therefore, Zn compounds at higher

concentration showed early senescence and greatly degraded or completely lysed bacteroids (**SI, Figure S9, C & D**). Additionally, the bacterial outer membrane protein plays an important role in early host recognition. Damages of rhizobial outer membrane affect the initial recognition, which resulted in the delay of the nodulation. Therefore, the infected cells in high concentration of treated nodules had relatively low bacterial occupancy and early senescence. However, the effect of NPs on bacterial outer membrane protein modification and host recognition mechanism need to be studied further in future.

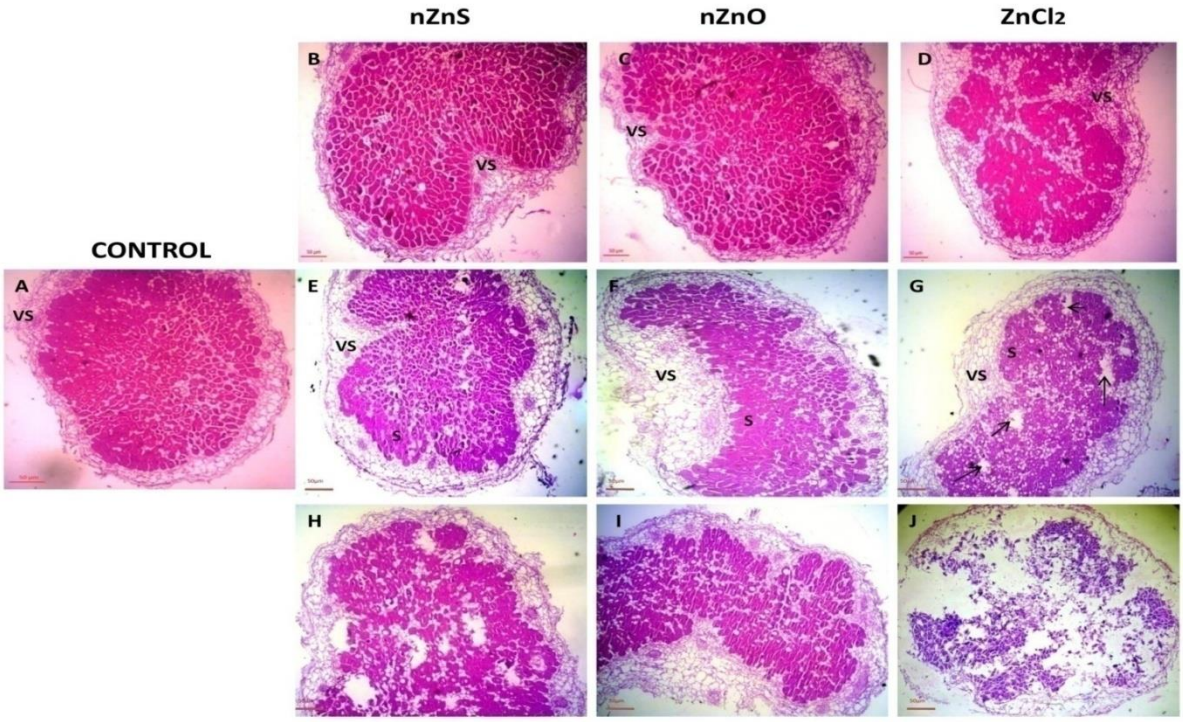

Figure 6. Low magnification images showing the morphology changes of (A) control, (B) 0.1 mg kg^{-1} nZnS, (C) 0.1 mg kg^{-1} nZnO, (D) 0.1 mg kg^{-1} $ZnCl_2$, (E) 1 mg kg^{-1}nZnS, (F) 1 mg kg^{-1}nZnO, (G) 1 mg kg^{-1} $ZnCl_2$, (H) 10 mg kg^{-1}nZnS, (I) 10 mg kg^{-1}nZnO and (J) 10 mg kg^{-1} $ZnCl_2$, nodules after 14 days of inoculation. (VS = vascular system, S = senescence zone, bar = 50µm).

4.4. Conclusion

Our results showed that NPs (nZnS and nZnO) performed better as source of Zn micronutrient than the ionic Zn i.e., $ZnCl_2$. NPs positively influenced mungbean growth at low concentrations. NPs treatments promoted plant growth and seed yield up to 0.1 mg Zn kg^{-1} whereas; phytotoxicity was observed when plants were grown in high concentration (10 mg Zn kg^{-1}) of NPs. Even the bacterial growth and outer membrane was damaged at high concentration of NPs. Therefore, the increase in biomass at low concentration suggested the optimum dose limit for the growth of mungbean plants. However, the decrease in biomass beyond this concentration suggested the toxic effect of NPs. $ZnCl_2$ showed highest degree of phytotoxicity. NPs did not cause toxicity different from that of $ZnCl_2$, which indicated that nZnS and nZnO used under the current experimental conditions did not cause nano specific risks. Moreover, NPs treatments alter plant micronutrient contents also. Significant translocation of Zn from leaf to fruit was found in NPs treated plants, compared to the $ZnCl_2$. Therefore, this result could overcome the problem of Zn deficiency in edible parts of plants. Also, in our study, nZnS worked as a better micronutrient than nZnO at low concentration due to their smaller size and slow release of Zn^{2+} ions. nZnS treatment resulted in the overall improvement in growth, including yield. Therefore, there exists an opportunity for nZnS to use as a suitable

alternative of commercially available bulk ionic salts for crop management at low concentration.

Supporting Information

Properties	Particulars	Values
Mechanical composition	Sand (%)	47.6
	Silt (%)	31.5
	Clay (%)	20.6
Chemical properties	Soil pH	7.59
	Organic carbon (%)	0.48
	Available nitrogen (kg/ha)	201.0
	Available phosphorous (kg/ha)	13.5
	Available potassium (kg/ha)	183.8

Table S1: Physico-chemical properties of the experimental soil (0-15 cm soil depth).

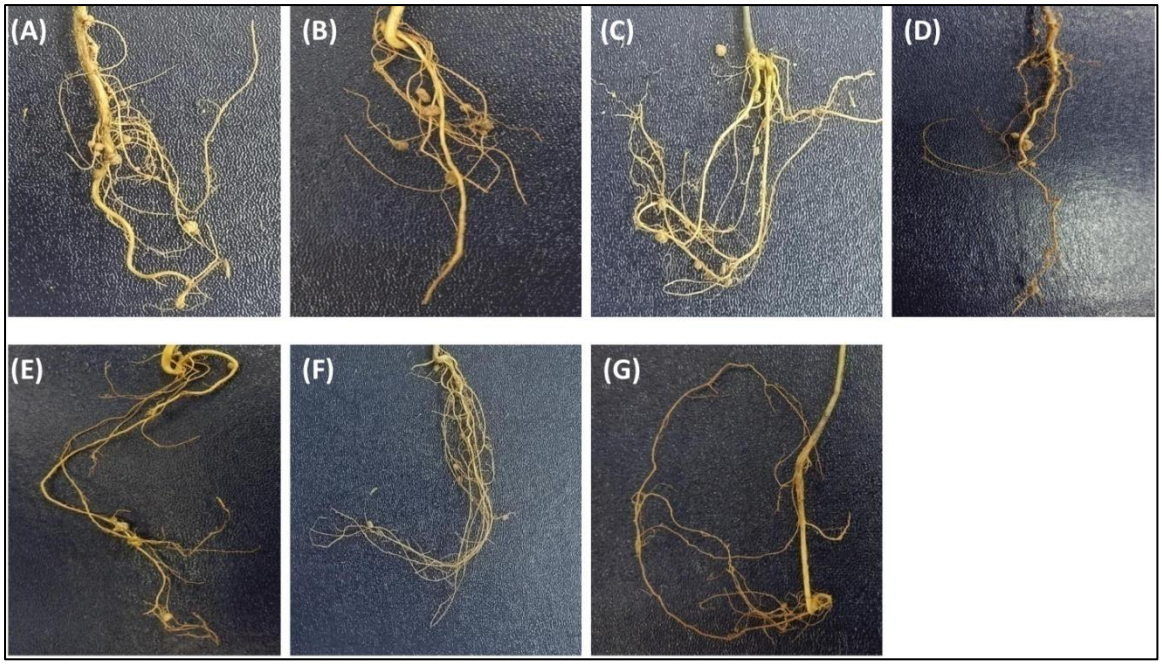

Figure S1: Morphology of roots after 60 days of growth in control and Zn amended soil; (A) control, (B) 0.01 mg kg^{-1} nZnS, (C) 0.01 mg kg^{-1} nZnO (D) 0.01 mg kg^{-1} $ZnCl_2$, (E) 10 mg kg^{-1} nZnS, (F) 10 mg kg^{-1} and (G) 10 mg kg^{-1} $ZnCl_2$.

Figure S2: Morphology of fruits after 60 days of growth in control and Zn amended soil; (A) control and (B) 0.1 mg kg^{-1} nZnS.

Treatments	Control	nZns	nZnO	$ZnCl_2$
Control	0.29±0.2 a			
0.01 mg kg^{-1}		0.44±0.03 b	0.41±0.02 b	0.25±0.01 a
0.1 mg kg^{-1}		0.45±0.03 b	0.42±0.2 b	0.19±0.02 c
1 mg kg^{-1}		0.38±0.05 b	0.36±0.03 b	0.19±0.02 c
0.01 mg kg^{-1}		0.37±0.1 b	0.36±0.01 b	0.19±0.02 c

Table S2: Translocation factors (TF) for Zn^{2+} from leaf to fruit (TF = C_{fruit}/C_{leaf}), ratio of concentration of Zn in leaf vs that in fruit. The TF are averages of 3 replicates ±SE.

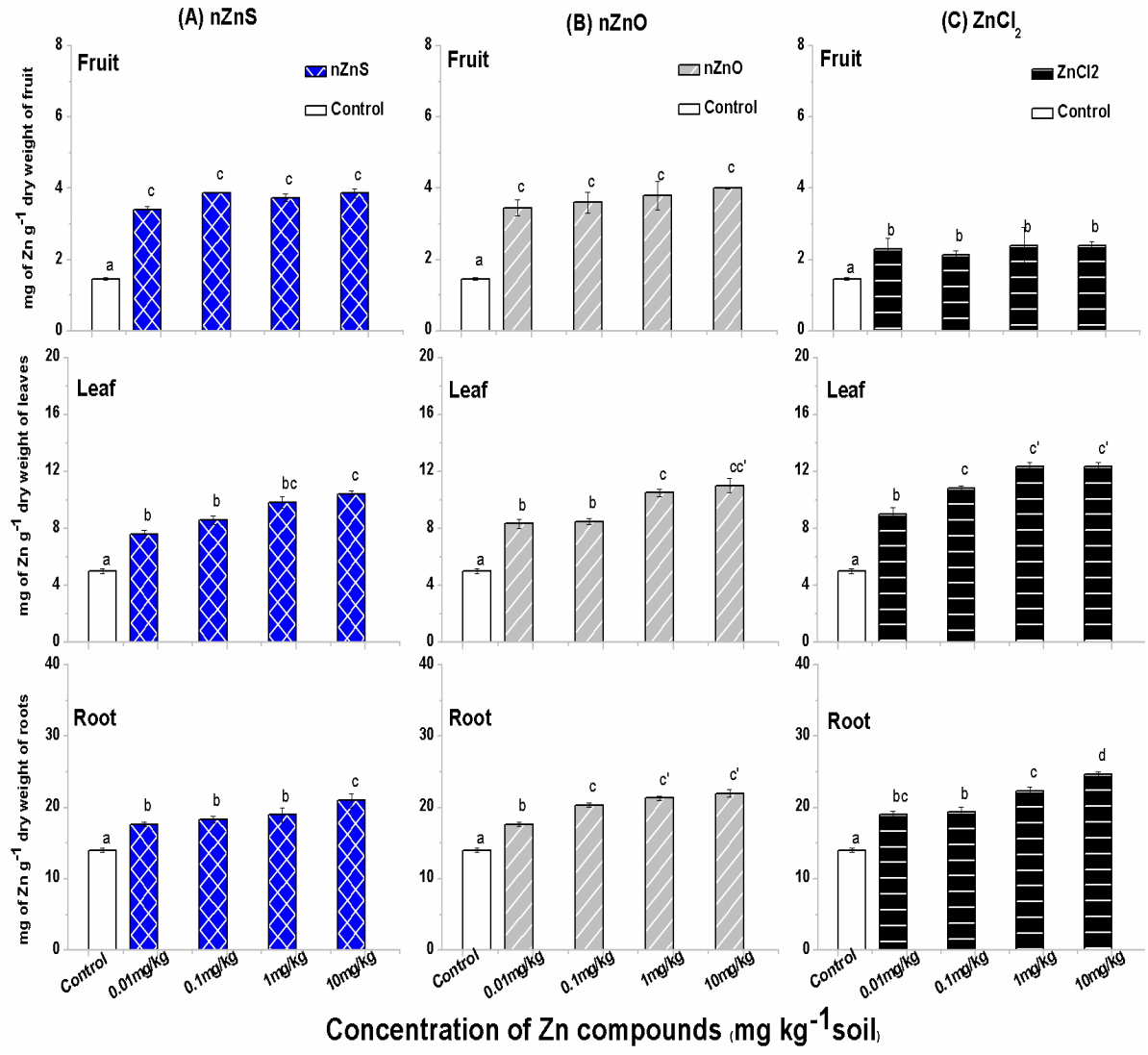

Figure S3. Zinc translocation in the fruit of mungbean plants cultivated for 60 days in soil amended with (a)nZnS, (b) nZnO, and (c) $ZnCl_2$ at 0 (control), 0.01, 0.1, 1 and 10 mg kg^{-1}soil. Data are averages of three replicates ± SE. Letter changes along the row depict significant difference, as per one-way ANOVA and the Tukey test ($p \leq 0.05$).

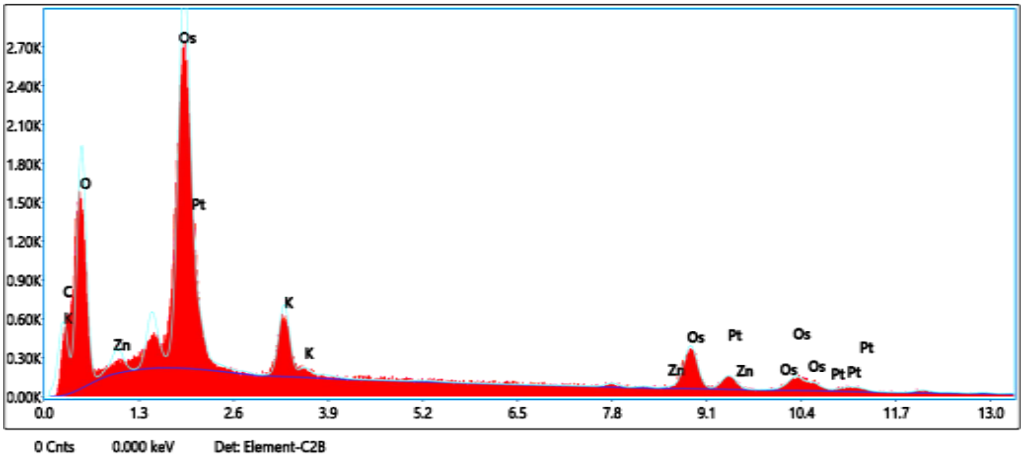

Figure S4. EDX image of cross section of root nodule of control.

Element	Weight %	Atomic %	Error%
C K	13.12	26.2	9.13
O K	43.58	65.32	9.42
K K	6.19	3.8	5.6
Zn K	0.19	0.07	73.14
Os L	22.11	2.79	10.38
Pt L	14.8	1.82	17.81

Table S3: EDX analysis of cross section of root nodule of control.

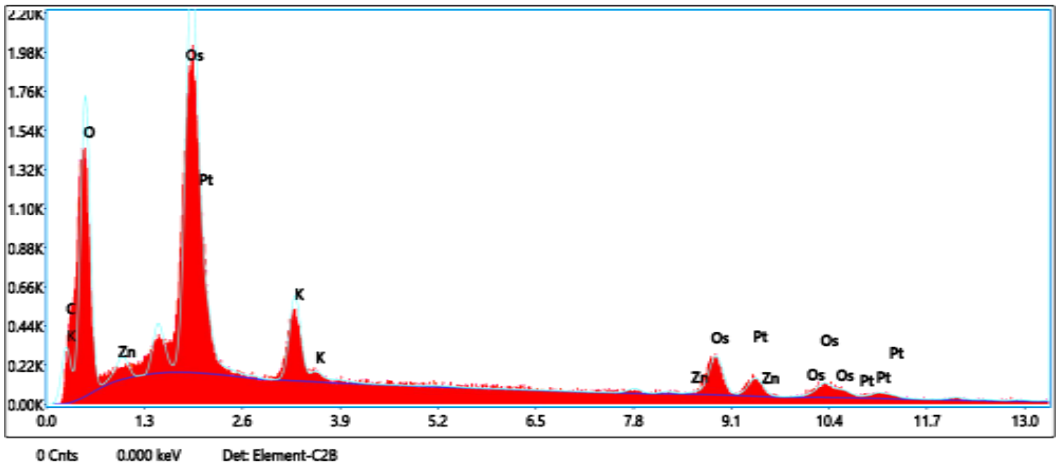

Figure S5. EDX image of cross section of root nodule of 0.1 mg kg^{-1} nZnS.

Element	Weight %	Atomic %	Error%
C K	9.7	21.6	9.95
O K	40.78	68.16	9.44
K K	5.96	4.07	6.26
Zn K	0.36	0.15	66.8
Os L	27.65	3.89	9.91
Pt L	15.55	2.13	18.08

Table S4: EDX analysis of cross section of root nodule of 0.1 mg kg^{-1} nZnS.

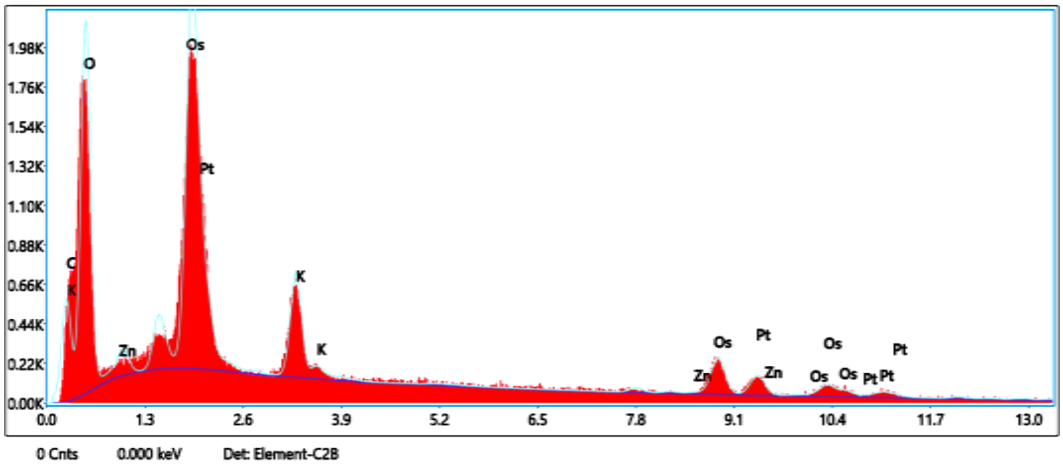

Figure S6: EDX image of cross section of root nodule of 0.1 mg kg^{-1} nZnO.

Element	Weight %	Atomic %	Error%
C K	14.15	30.31	9.37
O K	37.96	61.06	9.53
K K	4	2.63	7.16
Zn K	0.41	0.16	65.54
Os L	29.28	3.96	10.95
Pt L	14.21	1.87	20.54

Table S5: EDX analysis of cross section of root nodule of 0.1 mg kg^{-1} nZnO.

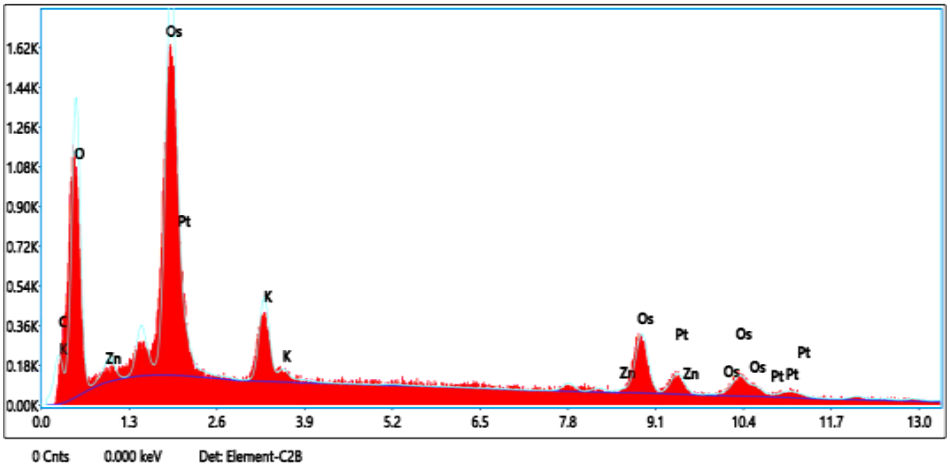

Table S7: EDX image of cross section of root nodule of 0.1 mg kg^{-1} $ZnCl_2$.

Element	Weight %	Atomic %	Error%
C K	6.75	17.52	11.14
O K	35.73	69.57	9.54
K K	5.4	4.3	6.88
Zn K	0.45	0.22	64.97
Os L	36.62	6	8.61
Pt L	15.05	2.4	22.93

Table S6: EDX analysis of cross section of root nodule of 0.1 mg kg^{-1} $ZnCl_2$.

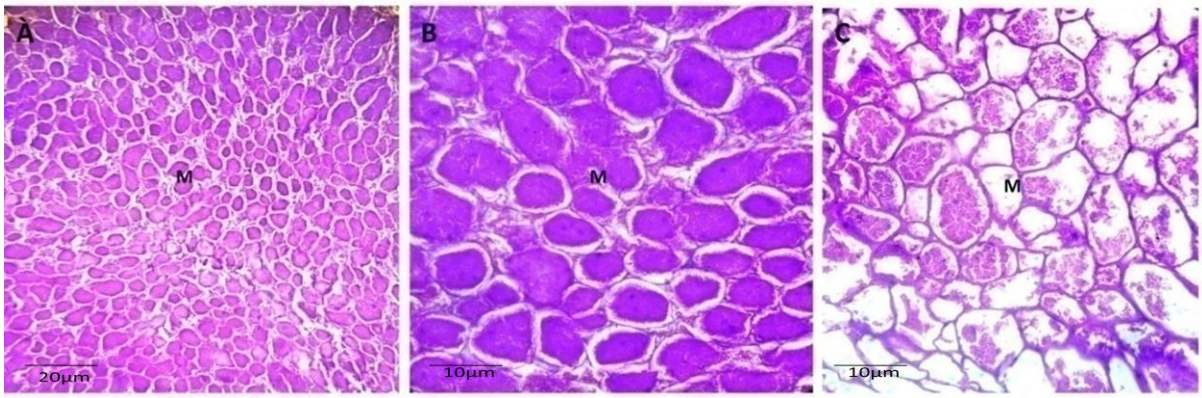

Figure S8: High magnification images showedinfected meristematic tissue (M) in the centre of a nodule primordium of (A) control (bar = 20µm), (B) 0.1 mg kg^{-1}NPs(bar = 10µm) and (C) 10 mg kg^{-1} NPs treated nodules (bar = 10µm).

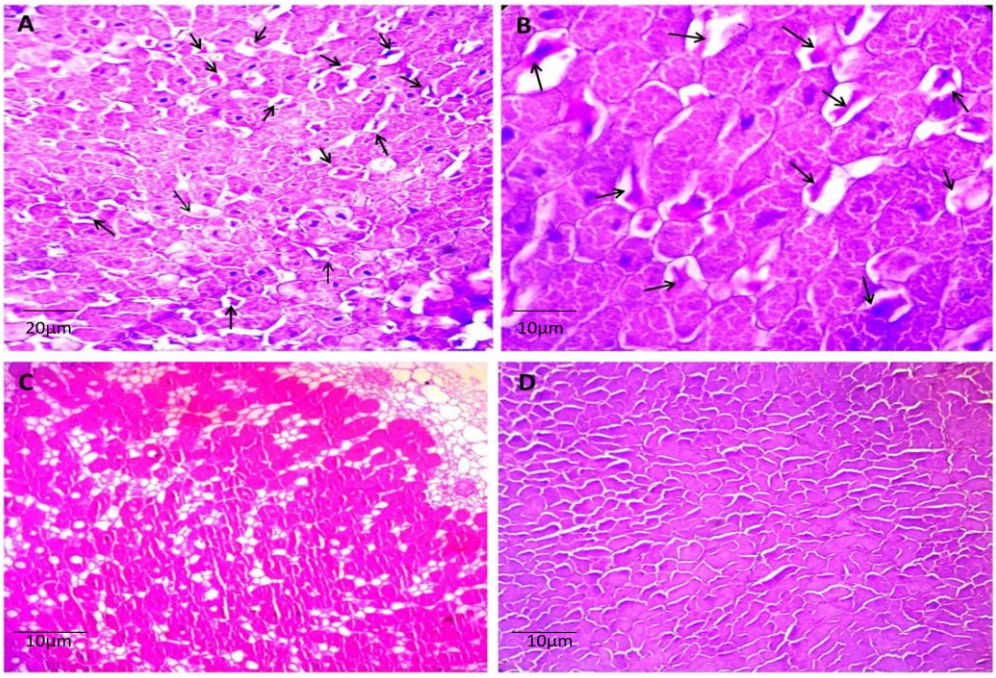

Figure S9. (A) Control (bar = 20µm) and (B) 0.1 mg kg^{-1} nZnS (bar = 10µm) nodule primordium, showed newly-infected cells containing newly released *Rhizobia* (arrow). (C) 10 mg kg $^{-1}$nZnS and (D) 10 mg kg $^{-1}$ $ZnCl_2$, showed abnormally disintegrated cells and a large area of senescence zone (bar = 10µm).

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CHAPTER 5

Conclusion and Future Direction

5.1 Conclusion

Overall this thesis investigated the effects of nZnS on growth, antioxidant defense system, ROS generation, nutritional status of plants, symbiosis, yield, and nutritional qualities of fruits of mungbean plants. In this thesis we introduced nZnS as a better alternative source of Zn micronutrient than nZnO and ionic Zn salt for agriculture and food sectors. This thesis works also aimed to overcome the Zn deficiency problem. In this thesis how the low concentration of nZnS enters inside the plants and remains in nano form was demonstrated. The work was aimed at understanding how the nano form of ZnS can change the different parameters of mungbean plants and promote growth. We presented the mechanism of nZnS translocation from roots to fruits. Effects of nZnS on Brady*rhizobium* symbiosis and nodule formation was also demonstrated and found that nZnS can be a suitable alternative of Zn micronutrient for crop plants.

5.2 Future Direction

Zn deficiency is one of the most widely distributed micronutrient problems limiting crop production in the world. In the agricultural sector, productivity depends on a large extent on agrochemicals, however conventional systems lack application efficiency, leading to environmental pollution, and related problems.

The use of nZnS can enhance Zn bioavailability by plants, in safer and affordable way. Future aspects in this project are to execute a systematic study of nZnS in various crop plants and to evaluate its effect in field conditions. We would also like to study the efficacy of the nZnS in the farm with farmers to understand its potential to become an alternative of Zn micronutrient in commercial markets.

PUBLICATIONS

Publications from Thesis Chapter:

1. **Mala Thapa**,* Tapodhara Datta Majumdar, Chandan Kumar Ghosh, Abhishek Mukherjee, and Prasanta Kumar Biswas. Application of Zinc Sulfide Nanoparticles to Augment the Nutritional Status of the Mungbean [*Vigna radiata* (L.) R. Wilczek] Plant. **ACS food science and technology (2021)**. https://doi.org/10.1021/acsfoodscitech.1c00116.

2. **Mala Thapa[a,b,c]**, Mukesh Singh[c], Chandan Kumar Ghosh[d], Prasanta Kumar Biswas[b], Abhishek Mukherjee[a,*]. Zinc sulphide nanoparticle (nZnS): A novel nano-modulator for plant growth. **Plant Physiology and Biochemistry 142 (2019) 73–83**. https://doi.org/10.1016/j.plaphy.2019.06.031.

Publication under Preparation:

1. **Mala Thapa[a,b,*],** Raghunath Sadhukhan[c], Abhishek Mukherjee[b], and Prasanta Kumar Biswas[a]. A Comparative Life Cycle Study of effects of nZnS, nZnO and $ZnCl_2$ on Mungbean Plant Yield and *Bradyrhizobium* Symbiosis.

Other Publications:

1. Tapodhara Datta Majumdara,[b,c,*], Mukesh Singh[c], **Mala Thapa[a]**, Moumita Dutta[d], Abhishek Mukherjee[a], Chandan Kumar Ghosh[b]. Size-

dependent antibacterial activity of copper nanoparticles against *Xanthomonas oryzae* pv. *oryzae* – A synthetic and mechanistic approach. **Colloid and Interface Science Communications 32 (2019) 100190**. https://doi.org/10.1016/j.colcom.2019.100190.

2. Khusbu Basumatary[a], Pallabi Daimary[a], Shon Kumar Das[a], **Mala Thapa[b]**, Mukesh Singh[b], Avik Mukherjee[a], Santosh Kumara,*. Lagerstroemia speciosa fruit-mediated synthesis of silver nanoparticles and its application as filler in agar based nanocomposite films for antimicrobial food packaging. **Food Packaging and Shelf Life 17 (2018) 99–106**. https://doi.org/10.1016/j.fpsl.2018.06.003.
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Book Chapters:

1. **Mala Thapa[a]** and Samrat Roy Choudhury[b,*]. Green Synthesized Nanoparticles: Physicochemical Properties and Mode of Antimicrobial Activities. **Comprehensive Analytical Chemistry (2021)**. DOI: 10.1016/bs.coac.2020.12.006.

2. **Mala Thapa*** 2015. UGC sponsored conference paper on Nanoparticle Exposure and its Impact on Environment and Human Health: A Literature Review. **ISBN 978-93-82623-51-1**. March 2015.

National/ International Conference Proceedings:

2. **Mala Thapa**, Abhishek Mukherjee, Prasanta Kumar Biswas, 2021. Oral presentation on "Application of Nanotechnology to Ameliorate Fortification and Yield of Crop Plants" WEBINAR organized by R&D Committee, TEQIP-III, Jadavpur University during February 26-27, 2021.
3. **Mala Thapa**, Abhishek Mukherjee, Prasanta Kumar Biswas, 2020. Oral presentation on "Fate of Zinc Nanoparticles in Plant Environment". National Conference on "Issues & Challenges in Water Treatment and allied research for Sustainable environment" during 23-25 Jan, 2020 at **IIT Guwahati**.
4. **Mala Thapa**, Prasanta Kumar Biswas, Abhishek Mukherjee, 2019. Changes caused by Zinc nanoparticles in lipid peroxidation and polyunsaturated fatty acids in mung bean plant. 6th India Biodiversity Meet 2019 (**International Conference**), held at Indian Statistical Institute, 203 Barrackpore trunk Road, Kolkata-700108, West Bengal, India. From 14-16th Feb, 2018.

4. **Mala Thapa**, Abhishek Mukherjee, Prasanta Kumar Biswas, 2018. Oral presentation on “Effect of ZnS nanoparticles on Overall growth and Antioxidant activity of *Vigna radiata* (Mungbean)”. National symposium on agricultural research under a changing climate in eastern India, organised by Agricultural and Ecological Research Unit, Indian Statistical Institute, Giridih, Jharkhand. January 2018.
5. **Mala Thapa**, Abhishek Mukherjee, Prasanta kumar Biswas 2018. Oral presentation on “ Root uptake and Dose dependent Phytotoxicity study of ZnS nanoparticles in *Vigna radiate*” in the 5th India Biodiversity Meet 2018 (**International Conference**), held at Indian Statistical Institute, 203 Barrackpore trunk Road, Kolkata-700108, West Bengal, India. From 15-17th March, 2018.
6. **Mala Thapa**, Mukesh Singh, Abhishek Mukherjee, 2015. Oral presentation on “Application of Nanotechnology in Agriculture” A Literature Review”. DST sponsored national seminar on New Horizons in Biotechnology organised by Department of Biotechnology, Haldia Institute of Technology, Haldia-721657, October 2015.
7. **Mala Thapa** 2015. UGC sponsored conference paper on Nanoparticle Exposure and its Impact on Environment and Human Health: A Literature Review. **ISBN 978-93-82623-51-1**. March 2015. (**Secured 3rd position in poster presentation**).

8. **Mala Thapa** 2013. Certificate of participation: Indian Biodiversity Meet-2013 organized by Agricultural and Ecological Research Unit, Indian Statistical Institute Kolkata in collaboration with Bio-mathematical society of India, 2013.

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Ms. Mala Thapa (born on 7th November 1988) completed her BSc in Botany from Bangabasi Morning College (University of Calcutta), in 2010. She topped the department in Bachelor's degree. She has completed her MSc in Botany from Bethune College (University of Calcutta), in 2012. She has done six months dissertation course in Nanotechnology in Agricultural and Ecological Research Unit, at Indian Statistical Institute (ISI), Kolkata, India. She worked as a Junior Research Fellow for 3 years in Agricultural and Ecological Research unit at ISI, Giridih unit, Jharkhand. She joined as a PhD scholar in Department of Food Technology and Biochemical Engineering at Jadavpur University, Kolkata, in 2015.