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EFFECTS OF COATED/UNCOATED COPPER-BASED NANOPARTICLES AND COMPOUNDS ON RICE (ORYZA SATIVA) AND SOYBEAN (GLYCINE MAX) PLANTS

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Dedication

This thesis is dedicated to my parents and my wife, for their unconditional love and support.

I admire and love you.

Even with all the hustle and hard times, they let me know I will never walk alone.
EFFECTS OF COATED/UNCOATED COPPER-BASED NANOPARTICLES AND COMPOUNDS ON RICE (ORYZA SATIVA) AND SOYBEAN (GLYCINE MAX) PLANTS

by

CHAOYI DENG, M.S.

DISSERTATION

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Abstract

Engineering nanoparticles (ENPs) have been widely used in industry due to their promising chemical, physical, electrical, magnetic, optical, and electronic properties. Copper-based ENPs are used as gas sensors, catalysts, semiconductors and agricultural products, among others. Because of their antifungal and antibacterial properties, they are used in agriculture as insecticides, herbicides and antifungal agents. Due to their mass production and use, ENPs are likely to be ubiquitous in our in the environment in the near future. In addition, metal nanoparticles are usually coated with organic compounds to enhance the properties of the nanoparticles. Previous studies have shown that copper-based ENPs can interfere with plant physiology and development. However, to our knowledge, very little work has been done on the effects of Cu-based NPs in rice (Oryza sativa) and soybean (Glycine max) plants. Rice is a very important crop widely consumed across cultures as a source of nutrition and caloric intake. Soybean is a worldwide consumed plant, which is very rich in protein. This research project is aimed to understand the effects of copper-based compounds on rice and soybean plants at full life cycle. It also analyzes the effects of surface coated copper based nanoproducts on soybean plants. The investigation included two parts. The first part consisted of culturing two type of rice plants (cultivated and weedy) until reaching physiological maturity (120 days), in soil amended with nano CuO (nCuO), bulk CuO (bCuO), and ionic copper (CuSO4) at 0, 75, 150, 300, and 600 mg/kg. Cu translocation, essential element accumulation, yield, sugar, starch, protein content, and the expression of auxin associated genes in grains were determined. The grains of weedy and cultivated rice were differentially impacted by CuO-based compounds. At ≥ 300 mg/kg, nCuO and bCuO treated rice had no grain production. Treatment at 75 mg/kg significantly decreased grain yield as compared to control with the order: bCuO (by 88.7%) > CuSO4 (by 47.2%) ~ nCuO (by 38.3% only in cultivated rice). These findings demonstrate a cultivar-specific and concentration-dependent response of rice to nCuO. A potential use of nCuO at 75 and 150 mg/kg in cultivar-dependent delivery system was suggested based on enhanced grain nutritional quality, although the yield was compromised. The second part
encompassed the evaluation of the effects that citric acid coated copper oxide nanoparticles (CuO-CA NPs) and their application process (foliar exposure, soil exposure) have on the growth and physiology of soybean (Glycine max). This part was conducted in two methods of application, soil exposure and foliar exposure. Seedlings (14-day-old) were transplanted into soil amended with 0 (control), 75, and 300 mg Cu/kg or foliar applied Cu-NPs suspensions. After nanomaterials exposure via foliar and soil application, Cu was detected in the roots, leaves, stem, pod, and seed of the plant. Cu was distributed differently depending on plant organs and surface coating. Foliar application of CuO NPs and CuO-CA NPs increased the soybean yield by 169.47% and 170.07%, respectively. In contrast, the ionic Cu treatment and soil exposure did not affect soybean yield. Our results show that CA coating changed the CuO NPs toxicity in soybean. This research provides direct evidence for the positive effects of CuO-CA NPs on soybean, including accumulation and transfer of the particles, which may have significant implications concerning the risk NPs pose to food safety and security.
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Chapter 1: Introduction

Nanotechnology, sometimes called molecular nanotechnology, has become one of the fastest developing industry trends in the 21st century. The global market is estimated to reach $22.3 billion in 2024 (Tewari and Baul, 2019).

Nanoparticles (NPs), as the basis of nanotechnology, are defined by the American Society for Testing and Materials (ASTM, 2006) as having a size of at least $10^{-9}$ meters (1 nm). Due to its small size and high surface area to volume ratio, compared with larger bulk particles, it is endowed with unique physical and chemical properties, such as higher chemical reactivity and conductivity, enhanced catalytic effect and physical strength, as well as unique magnetic and optical effects (Zuverza-Mena et al., 2017). Engineered nanoparticles (ENPs) with these properties are designed for special applications. ENPs enhanced biocompatibility and reactivity enable it to be integrated into the fields of industry, medicine, consumer goods and energy. (Abdussalam-Mohammed, 2019; Ahmadi et al., 2019). ENPs also have broad potential application prospects in the field of agriculture (Chhipa, 2019; White and Gardea-Torresdey, 2018). Nano-elements can be used as fertilizers or pesticides to provide nutrition and resist plant pathogens in a variety of plant species (Huang et al., 2015; Liu and Lal, 2015). Compared with traditional products, nano pesticides and nano fertilizers may be more effective and may reduce the application rate (Kah, 2015).

Nevertheless, many studies have reported ENPs induced toxicity. After use, a large amount of ENPs is directly or indirectly released into the environment. Soil and water are considered to be the largest sinks of ENPs. This may threaten agricultural ecosystems. Terrestrial ecosystems are most affected because soil is one of the largest sinks of all released NPs. Plants are the main food source of organisms and interact directly with the released NPs. Any impact of NPs on plants will
eventually affect the food chain, including humans, because humans are the final consumers. It is necessary to understand the various effects of ENPs exposure to plants, develop safety protocols for NPs application and disposal in agriculture, and implement appropriate supervision.

By 2010, the annual production of copper oxide NPs (nCuO), one of the most widely used copper based material, has exceeded 200 metric tons (Keller et al., 2013). CuO based nanoparticles have been widely used in batteries, polymers, lubricants, gas sensors, pigments, and catalysts (Anjum et al., 2015). In addition, the production and use of nCuO in agricultural systems are increasing (European Commission, 2018). In order to improve performance and meet the needs of various customers, surface modified nCuO has been widely used to achieve better appearance, performance or advanced functions in different types of coatings, such as corrosion resistance, less catalytic degradation, self-cleaning and decontamination (Bergamonti et al., 2015; Calia et al., 2017; Chen et al., 2019; Colangiuli et al., 2019). Based on its exponential production, it is expected that more coated nanomaterials will be released into the environment on a large scale (Keller and Lazareva, 2014). The estimated emissions from soil and water in 2010 were as high as 23,130 and 36,664 metric tons/year, respectively (Keller and Lazareva, 2014). Moreover, some wastewater and sewage sludges containing CuO might be used to treat agricultural soils (Cornelis et al., 2014). Due to various soil components, including humus and non-humus compounds, microbial community structure and soil ionic strength, the interaction between coated nCuO and soil is complex (Fig. 1) (Rawat et al., 2018). Therefore, the impact of coated ENPs on plants will depend on several factors, which include plant species, surface coating properties (surface area, charge, hydrophilicity, and suspension stability), growth stage, soil type, and exposure method (Tan et al., 2018).
Previously, various studies have been conducted to investigate the effect of exposing nCuO and coated nCuO to terrestrial plants. The potential impact of nCuO application in the agriculture field is proposed. In the greenhouse experiment, 1-2 mL of 500 mg/L nCuO suspension was foliar applied to the 30-day-old tomato (Solanum lycopersicum) seedlings and promoted plant growth (Ma et al., 2019). In another full life cycle study, two type of bok choy (Rosie and Green) were suppressing by nCuO ((Deng et al., 2020). A similar suppression effect was also found in watermelon (Citrullus lanatus). Meanwhile, Wang et al. suggested that nCuO be used as a potential agricultural fertilizer in the growth of lettuce (var. ramosa Hort.) (Wang et al., 2019). The results showed that nCuO treatments increased above-ground biomass of lettuce (16.3–19.1%), while bulk CuO did not. It may be due to the enhancement of plant photosynthesis system, the increase of transpiration rate and stomatal conductance.

Cultivated rice (Oryza sativa L.) and weedy rice (Oryza sativa f. spontanea Rosh.), are essential crop in worldwide. It is a staple food for more than 50% of the world’s population, and 90% of the world’s rice grows from Asia (Nadir et al., 2017). They have a high concentration of starch, protein, as well as fiber. Weedy rice, one of the biggest problems of global rice cultivation. They compete with cultivated rice to grow in the field. Because of its short growth cycle and resistance to herbicides, it suppresses the growth of cultivated rice. Weedy rice can also be used as an important genetic resource to improve the performance of cultivated varieties (Chauhan, 2013). However, the weedy rice is not a very well-studied species in the rice family. The effect of nCuO exposure on the growth of weedy rice is unknown.

On the other hand, soybean (Glycine max) are economically relevant crops that contain high nutritional value. Their 2018 annual total production was >119 million metric tons in the United States. They have a high concentration of protein (18%) (Wei and Molin, 2020). The beans
are the main consumed portion of soybean. Since they are indirect contact with soil, they will interact with the foliar applied ENPs. Thus, there is a need to investigate the effects of different application methods of ENPs on the crops (Peréz et al., 2020). Moreover, coated ENPs and bare ENPs have many different properties; currently, the studies evaluating the effects of coated CuO ENPs on soybean are very limited. Additionally, the applied method effects of surface-coated nCuO on plant development are still undescribed.

Two-photon microscope is gaining more and more attention in studying the fate of ENPs (Deng et al., 2020). Compared with other microscopy techniques such as transmission electron microscopy (TEM) or scanning electron microscopy (SEM), the two-photon microscopy has the advantage of penetrating the sample and observing the internal structure through fluorescence. Additionally, samples are easy to prepare and when operated at room temperature there is no further damage during the detection process. Since nCuO have self-fluorescence, no further dying process is needed, which makes nCuO a good candidate for two-photon microscopy studies.

In this work, there were two study phases. In phase I, the physiological and biochemical effects of nCuO on rice plant was studied. Rice was harvested after growing for 120 days in soil treated with nano (nCuO), bulk (bCuO), and CuSO₄ at 75–600 mg/kg. In phase II, the effects of citric acid surface-coated nCuO on soybean plant development were investigated. Soybean were treated with soil and foliar exposure nCuO and citric acid coated nCuO at 75, and 300 mg/kg for 65 days until full-plant maturity. In all phases, the agronomic parameters of plant growth and essential element accumulation were determined. The penetration and uptake of nCuO were obtained by inductively coupled plasma-optical emission spectroscopy (ICP-OES) and two-photon microscopy.
Fig. 1.1 Factors affecting the fate and transport of ENPs in soil. *Reprint from* Rawat et al. (2018)

**General Objectives**

1. Evaluate and compare the different physiologic and biochemical effect of three Cu-based compounds (nano CuO, bulk CuO and CuSO₄) on two types of rice plants grown to the mature stage.

2. To investigate the surface coating effect of nano CuO on the physiological and biochemical responses of soybean plants.

3. To study the applied method effect of nCuO on soybean plant development.
Research Questions

1. How will the nutrient content in rice and soybean be affected by nCuO exposure? Does size-different really effect the Cu uptake in plants?

2. Does application method effect nanoparticle uptake? Is foliar application better than soil exposure?

3. Will the citric acid coated-nano CuO undergo the same uptake process? Will surface coated-nano CuO differently affect plants?

4. Is it necessary to regulate the use of nCuO/coated nCuO? Do they have potential applications in agriculture?

Hypothesis

1. The exposure of nCuO will suppress the development of weedy rice plants.

2. The nano CuO with the surface coating will be differently uptaken by soybean and will affect soybean development in particular forms.

3. The foliar exposure application will change the initial physiological and biochemical responses of plants exposed to soil exposure ENPs.
Chapter 2: Copper oxide (CuO) nanoparticles affect yield, nutritional quality, and auxin associated gene expression in weedy and cultivated rice (Oryza sativa L.) grains

2.1 Introduction

With an estimated population of over 9 billion by 2050, the potential use of engineered nanoparticles (ENPs) to promote an increased yield of crops has been explored as a means to maintain global food security. Copper oxide nanoparticles (nCuO) are among the most widely used ENPs, with an emerging promissory applications in agriculture (Hofmann et al., 2020; Kah et al., 2019). Recently, Cu based nanomaterials have been investigated as potential nanofertilizers or nanopesticides to increase crop production, improve edible tissue nutrition, and suppress diseases (Dimkpa et al., 2019; Elmer et al., 2021; Ma et al., 2020; Shang et al., 2021; Shen et al., 2020; Wang et al., 2020). Although promising results have been published, the absorption or uptake of nanoscale CuO into plants may occur and its transfer to sensitive receptors, including humans, needs to be thoroughly characterized.

Although the phytotoxicity of nCuO on plant species has been reported, there is still a debate over the extent of toxicity directly from nanoscale particles and that from released Cu ions. Ameh and Sayes (2019) reported that the physicochemical properties of nCuO (size, surface functionalization, solubility, among others) affected the oxidative stress level, bioactivity, and bioaccumulation of Cu in plants. Joško et al. (2021) reported that the biomass of barley (Hordeum vulgare L.) decreased after being treated with nCuO at 300 mg/kg soil for 7 days; meanwhile, a joint exposure of nanoscale CuO and ZnO resulted in a similar reduction of ~ 25% (Joško et al.,
For Rosie and Green bok choy (*Brassica rapa var. chinensis*) plants, Deng et al. (2020) reported significant variety-dependent Cu distribution and growth inhibition in response to nanoscale and bulk CuO at 75-600 mg/kg soil (Deng et al., 2020a). Notably, Rosie, with higher anthocyanin content, was more vulnerable to nCuO-induced stress. Peng et al. (2017) reported that plant length and biomass of rice cultivated in soil were negatively affected by exposure to nanoscale CuO. At 1000 mg/kg, only one rice plant survived; at 500 mg/kg, the fresh weight of treated grains was 6.51% of the controls (Peng et al., 2017). Rai et al. (2021) reported that exposure to 100 μM nCuO under hydroponic conditions decreased the growth of fifteen day-old rice seedlings and induced oxidative stress (Rai et al., 2021).

Weedy rice (*Oryza sativa f. spontanea* Rosh., commonly known as red rice) has evolved from wild rice (*Oryza rufipogon*) and cultivated rice (*Oryza sativa* L.), and its presence is a significant problem for global rice cultivation. Weedy rice is characterized by aggressive growth, early flowering, heavy seed shattering, and evolved resistance to herbicides, all of which enable it to outcompete cultivated rice in the field (Nadir et al., 2017). This competition typically reduces the total yield and the quality of the product, generating undesirable brown grains. As estimated, the simultaneous weedy rice infestation in Asian countries could reduce 1.9 million tons (0.5%) in long-grain rice output (Juliano et al., 2020). Conversely, weedy rice could also serve as an important genetic resource to improve the performance of the cultivated species, for instance, by enhancing disease resistance (Zhang et al., 2019), promoting growth and photosynthetic rates (Thurber et al., 2013), and overcoming seed shattering and dormancy issues (Subudhi et al., 2014, 2012). Genetic studies have revealed the evolutionary relationship between the ancestral weedy rice and cultivated rice (Subudhi et al., 2020, 2018) and several genome-wide analyses have further
confirmed their possible origins (Chai et al., 2018; He et al., 2017; Li et al., 2017; Qiu et al., 2017; Tong et al., 2017).

Studies evaluating the impact of nCuO on crop yield and grain nutritional quality after full life cycle exposure under field conditions are scarce (Wang et al., 2021c). Importantly, although previous work has shown that ENPs can affect plant development at the genetic, physiological, and biochemical level, few studies have investigated related responses between two plant cultivars sharing homologous genes. Here, two rice varieties (cultivated and weedy) were grown in a field soil amended with nanoscale CuO (nCuO), bCuO, or CuSO₄, individually at 0-600 mg Cu/kg soil. After a full life cycle exposure, the developed grains were collected and Cu uptake and translocation was quantified. The biodistribution of Cu-based particles in grains was observed by two-photon microscopy. Grain yield, as well as the content of elemental nutrients, sugar, starch, and protein was determined. The growth-related genes transcription was determined. This study facilitates the understanding of the mechanistic interactions of nCuO with two genetically related but different model plant species, which promotes the development of sustainable nano-enabled agriculture.

2.2 Materials and Methods

2.2.1 Soil and Cu Based Treatment Preparation

Copper sulfate (CuSO₄) and bulk size CuO (bCuO) were purchased from Sigma Aldrich (St. Louis, MO). Copper oxide nanoparticles (nCuO) were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC-CEIN). The characterization of nCuO and bCuO has been described in previous studies and is shown in Table
The effect of pH (ranged from 3-10) on the $\zeta$-potential of nCuO and bCuO suspended in DI at 300 mg/kg are shown in Fig. S2.1 (Deng et al., 2020a). Miracle-Gro® organic potting mix was purchased commercially. A field soil (medium loam) was collected from an agricultural area in Socorro TX, USA, at latitude: 31°67′ N, longitude: 106°28′ W, and elevation: 1115 m asl. The properties of the field soil were: 2.8% organic matter, pH = 7.8 ± 0.02, EC = 1705 ± 47.6 µS cm-1, and TDS = 847.5 ± 23.8 mg/L (Majumdar et al., 2015; Wang et al., 2020). The soil was sieved through an eight-mm sieve and air-dried for two days to remove residual plant debris and gravel. To ensure optimum plant growth, the field soil and potting soil were mixed at a ratio of 2:1 by mass.

To prepare suspensions/solutions of each Cu-based compounds, nCuO, bCuO, and CuSO$_4$ were weighed according to the total amount of Cu needed in each pot, and amended in 50 mL Milli-Q 18 MΩ deionized water (DI). The solutions were sonicated in a water bath at 25 °C for 30 min at 180 watts. For each replicate pot, 6 kg of the soil mixture was amended with the above suspension/solution and mixed manually for at least 20 min to ensure homogeneity. For the controls, 50 mL of DI was used. The final concentrations were 75, 150, 300, and 600 mg Cu content/kg soil; this dose range was selected based on environmental relevance and the existing literature (Jiang et al., 2021; McGrath et al., 1994; Peng et al., 2020; Wang et al., 2020). Each of the pots was placed into a larger plastic container to prevent leachate loss and to provide sufficient moisture for rice growth. Pots were then brought to 60% of the maximum field moisture capacity with DI water and left for three days before use to equilibrate.

2.2.2 Greenhouse Cultivation

Cultivated (Oryza sativa subsp. indica) and weedy (Oryza sativa f. spontanea Rosh.) rice seeds were provided by the Louisiana State University Agricultural Center (Baton Rouge, LA,
USA). The seeds were sterilized with NaClO and sowed in Metro-Mix 360 (SunGro Hort., Bellevue, WA). After 21 days of germination, uniformed seedlings were transplanted into each pot filled with the prepared soil (described above) and placed in the greenhouse of Texas A&M AgriLife Research Centre at El Paso (TX) under a well-controlled condition as described before (Deng et al., 2020; Wang et al., 2020). Briefly, the average temperature of day/night was 30/20 °C, with a 50-75% relative humidity and light daily integral at ~10 mol m\(^{-2}\) d\(^{-1}\). One seedling was planted in each pot, with four replicate pots for each treatment. Plants were fertilized with Yoshida's nutrient solution every other week. A completely randomized design factorial was adopted for the arrangement of pots. Grain samples were collected after 4 months of transplanting when all the plants were fully mature. All grain tissues were weighed, frozen in liquid nitrogen, and stored in a -80 °C freezer.

2.2.3 Elemental analysis

Macronutrient (Ca, Mg, P, S, and K) and micronutrient (Cl, Zn, Fe, B, Mn, Cu, Mo, and Ni) tissue content was measured by inductively coupled plasma – optical emission spectrometry (ICP-OES, Perkin-Elmer Optima 4300 DV; Shelton, CT). At harvest, plants were separated into grain and spike. Each sample was oven-dried at 70 °C for 72 h. A coffee grinder (Hamilton Beach) was used to homogenize dry samples. Approximately 0.2 g of dry tissues was amended with 2 mL of pure trace HNO\(_3\) (SPC Science, Champlain, NY); the samples were acid digested at 115°C for 45 min on a Digiprep hot block (SCP Science). The samples were then amended with 1 mL of 30% hydrogen peroxide and heated for an additional 20 min to complete the digestion. The digests were adjusted to 45 mL with DI. Blanks (no plant tissues), a standard reference material (peach leaf, 1547, National Institute of Standards and Technology, Gaithersburg, MD) and spiked samples were
also digested by the same procedure to ensure the adequacy of method performance. The multi-elemental standard solution was evaluated every 20 samples.

2.2.4 Two-photon microscopy

For two-photon microscopic imaging, fresh roots from cultivated and weedy rice treated with 0 or 150 mg/kg Cu-based compounds were collected after 4 months of growth. Grain were washed three times with DI water and 0.01% nitric acid and then cut by a CryoMicrotome (Triangle Biomedical Sciences, Durham, NC) (Wang et al., 2021b); the sections were imaged with a custom-built two-photon microscope following established method (Acosta et al., 2014; Deng et al., 2020a; Wang et al., 2020). The light source is a mode-locked Ti: Sapphire laser (Maitai HP, 100 fs, 80 MHz, Spectra-Physics). A 665 nm long-pass dichroic mirror deflects fluorescence signals to photomultiplier tubes (PMTs) for detection and a personal computer acquires signals from PMTs to form images in the x-y plane. Pure suspensions of nCuO, bCuO, and CuSO₄ solution in DI water were also analyzed for comparison. A wavelength of 710 nm and laser power of 200 mW was used for two-photon excitation analysis (Acosta et al., 2014; Deng et al., 2020a; Wang et al., 2020).

2.2.5 Grain macromolecular nutrient

Protein content-The determination of protein content was conducted according to the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). First, one mL of DI was added to 20 mg of each dry sample powder. The homogenate was vortexed for 2 minutes and stored overnight at 4°C. Then 150 µL of the sample solution was added into microplate wells, followed by 150 µL of the Bradford reagent. The mixture was shaken for 30 seconds and then incubated at room temperature for 10 minutes in darkness. Bovine serum albumin (BSA) protein ranging from 0 to
25 µg/mL was used to generate the standard curve. After incubation, the absorbance was measured at 595 nm on a UV-Vis microplate plate reader. Protein content is expressed as µg/mL.

Sugar and Starch-The content of total sugar and starch were measured according to a previous assay with some modifications (DuBois et al., 1956). Ten mL of 80% ethanol was added into ~100 mg dry grain powders. Homogenates were heated in an 80 °C water bath for 30 min, and centrifuged for another 20 min at 22,000 × g. These extraction steps were repeated three times and all replicate supernatants were mixed. The residues were also collected and kept at 80 °C after drying for 24 h. To determine total sugar content, each ethanol extract was partially evaporated to 3 mL and then diluted to 25 mL with DI. For starch measurement, dry residues were amended with 2 mL DI and samples were added to boiling water for 15 min. After cooling to ambient temperature, 2 mL of concentrated H₂SO₄ was added. After incubation for 15 min, tubes were brought to 10 mL with DI and centrifuged at 3000 × g for 20 min. The extraction was repeated using 50% of H₂SO₄. Both supernatants were recovered, mixed, and brought to 50 mL with DI. The concentrations of total sugar and starch were quantified according to DuBois et al. (1956) using standard curves.

2.2.6 RNA Isolation and RT-qPCR

Frozen grain samples were ground in liquid nitrogen. PureLink™ Plant RNA Reagent (Invitrogen™) was used to extract their total RNA followed by a purification procedure according to the protocol provided by the manufacturer. The final quality and concentration of RNA were examined using a NanoDrop spectrophotometer (Thermo Scientific). To perform the RT-qPCR analysis, cDNA was firstly synthesized from the RNase-free DNase I (Roche)-digested RNA using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ThermoFisher Scientific). Then quantitative PCR (qPCR) was performed in a StepOne™ Real-Time PCR system with iQ™ SYBR® Green Supermix (Biorad). The final concentration of cDNA was 5 ng/mL. The
selected genes and their corresponding primers are: copper/zinc-superoxide dismutase gene (Cu/ZnSOD), forward primer 5’-TTTGCTCAAGAGGGAGATGG-3’ and reverse primer 5’-TTGTAGTGTGGCCCATTTGA-3’; auxin response factor gene associated with plant growth, forward primer 5’-GGAGGAGGAGGAGGAGGAGG-3’ and reverse primer 5’-TGCGGGAAGTAGAACACCTT-3’; and the housekeeper glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, forward primer 5’-GAAGCACAGCGACATCAAGA-3’ and reverse primer 5’-CATACTCAGCACCAGCCTCA-3’. Quantified gene expression was calculated according to the 2ΔΔT method (Cota-Ruiz et al., 2020).

2.2.7 Statistical analysis of the data

Statistical significance was determined at p ≤ 0.05 by the Statistical Package for the Social Sciences 25 (SPSS, Chicago, IL, USA) using one-way ANOVA followed by the Tukey–Kramer multiple comparison tests. Data are expressed as the mean ± standard error (SE) of three replicates in tables/figures.

2.3. Result and Discussion

2.3.1 Cu uptake and translocation

Representative two-photon microscopy images of rice grains exposed to nCuO and bCuO presenting the translocation and biodistribution of CuO-based compounds are shown in Fig. 2.1 Cu was found in grains of both cultivated and weedy rice treated with nCuO and bCuO at 75 mg/kg. The characterization of pure nCuO and bCuO suspensions in DI water is presented in Supplementary Fig. S2.2 Specific detection parameters for these compounds were previously set (Deng et al., 2020a; Wang et al., 2020). No fluorescent signal was observed from the CuSO₄ DI
solution or the two untreated weedy and cultivated grain controls. In Fig. 2.1, representative fluorescent spots of Cu are pointed out by arrows, showing the distribution of Cu signals from nCuO and bCuO exposure in grains, indicating the translocation of nCuO and bCuO from root to grain which is the edible part of rice. This also shows that higher copper concentration is present in both rice varieties treated with nCuO when compared to the bCuO treatment.

As shown in Fig. 2.2A, treatment with nCuO (at 75 and 150 mg/kg) and ionic CuSO$_4$ (at all concentrations) significantly increased Cu accumulation in the rice grain as compared to controls; interestingly, no such increases were evident with bulk CuO, demonstrating a size-dependent effect for CuO. Specifically, the Cu grain concentration in the control, 75 and 150 mg/kg treated soils were 9.97, 15.4 and 19.8 mg/kg, respectively; treatment at 75 and 150 mg/kg resulted in 54.1% and 98.3% increases in Cu content, compared to controls (p ≤ 0.05) (Fig. 2.2A). The Cu increase in CuSO$_4$-treated plants was 58.2% compared to the untreated controls (p ≤ 0.05). While comparing the two rice varieties, weedy rice favored Cu accumulation in grain when treated with nano CuO or CuSO$_4$, over the cultivated one. After normalization to the respective controls, the increase of Cu in weedy rice grains exposed to nCuO and CuSO$_4$ was 2.56- and 3.10-fold greater than in the cultivated rice (p ≤ 0.05). The Cu content of the spike tissue is shown in Fig. 2.2B. Not surprisingly, significantly greater Cu accumulation was found in nanoscale and bulk CuO treatments (up to 17.6 mg/kg) as compared with controls (6.40-8.57 mg/kg) (p ≤ 0.05); however, there were no differences as a function of Cu or rice type.

The finding that nanoscale CuO leads to greater Cu grain content than did bulk Cu is not surprising. According to a previous dissolution study, after 48 hours, the concentration of Cu$^{2+}$ released from 500 mg/kg bulk Cu in soil solution was approximately 40 µg/L (Rawat et al., 2019). At the same concentration, nanoscale Cu yielded a much greater Cu ion dissolution (up to 100
µg/L) in soil solution after 48 hours as compared to bulk Cu (Rawat et al., 2019). Although the current study was done in soil, it is clear that this relatively low amount of Cu ion (100 µg/L) was insufficient to induce a significant increase of Cu content in grain. In addition, nCuO with much smaller size was able to more readily enter into plant roots and translocate through the xylem system (Deng et al., 2020a; Wang et al., 2020). This trend has been reported in media other than soil, confirming that the smaller size of nCuO favors Cu release, although there is a balance of enhanced dissolution and biomolecule attachment (i.e., corona) as a function of small size. For example, a significantly greater release of Cu from nCuO than bCuO was reported in Luria-Bertani medium (Gunawan et al., 2011). McShane et al. (2014) reported that nCuO with a much larger specific surface area might result in more significant interaction with soil organic matter or the soil microbiome as compared to bCuO, changing its bioavailable fraction in the rhizosphere. It is also possible that given the nanoscale size, nCuO could directly enter the plant and be subject to root-to-shoot translocation. Notably, several condition-based transformation processes such as dissolution, aggregation, and dynamic corona formation would impact CuO fate in unknown ways (Borgatta et al., 2021; Deng et al., 2020a; Wang et al., 2020).

2.3.2 Grain yield, percentage of full grains, full grain average weight, and spike biomass

The grain production of cultivated and weedy rice was 29.1 and 20.4 g/plant, respectively. The reduction of grain production caused by Cu-based compounds was significant compared with untreated controls. As shown in Fig. 2.3, plants exposed to nanoscale CuO at 75 and 150 mg/kg produced 38.4% and 64.6% of the controls. The greatest reduction was with bulk CuO treatments, particularly in cultivated rice at 150-600 mg/kg, where all the production was lost. Both rice types had greater tolerance/survivability to CuSO₄ than to nanoscale or bulk CuO; grains were still developed in CuSO₄ treated plants at up to 300 mg/kg for cultivated rice, and up to 600 mg/kg for
weedy rice. Interestingly, no significant differences were observed in responses between cultivated and weedy rice to all the Cu-based treatments. The Cu-based compounds also decreased spike biomass, although this did not vary with rice type. Exposure to 75 or 150 mg/kg nanoscale CuO reduced spike mass by up to 52.5%; bulk CuO at 75 mg/kg reduced spike mass by 38.7%. Interestingly, no negative response was found at 75 mg/kg nanoscale CuO treatments in both cultivated and weedy rice, suggesting a dose-dependent toxicity as well as a potential threshold of 75 mg/kg for the potential application of nano CuO in agricultural soils. However, this could clearly be impacted by soil type, pH, microbiome characteristics and background Cu levels. It has been reported that an aging process in soil can alleviated the original NPs toxicity to crops (Wang et al., 2021a).

Unlike total grain biomass, no significant decrease in the percentage of full grains (PFG) or full grain average weight (FGAW) was observed, regardless of Cu or rice type (Fig. 2.4). In fact, weedy rice exposed to 75 and 150 mg/kg of bCuO, and 150 mg/kg of nCuO exhibited significantly increased PFG and FGAW values compared to untreated controls (p ≤ 0.05); interestingly, no such improvement was evident in cultivated rice. Importantly, at 150 mg/kg, nCuO and bCuO increased FGAW in weedy rice by 96.0 and 221.2%, respectively.

Given these results and the Cu accumulation data presented above, it is clear that CuSO₄ led to a significantly higher Cu uptake into the grains but caused much less yield loss than bCuO. The explanation for this lack of correlation between grain Cu content and yield is not known. Previous studies suggest that the mechanism of nCuO phytotoxicity on rice was associated with an excess formation of reactive oxygen species (ROS) and subsequent disruption in the transcription of related and important genes (Wang et al., 2015, 2021c, 2020). Shaw and Hossain (2013) reported rapid cell death in rice seedlings exposed to 1.5 mM nCuO for 7 and 14 days under hydroponic
conditions and linked this response directly to ROS stress (Shaw and Hossain, 2013). Yang et al. (2015) exposed rice to 1000 mg/L nCuO for seven days under hydroponic conditions and reported that the growth of rice seedlings had nearly ceased and a reduction of root elongation of 97% with respect to the controls was noted (Yang et al., 2015). Notably, previous studies have also demonstrated that the negative effect nCuO is highly concentration-dependent, although the vast majority of the reported studies were conducted under hydroponic conditions. Tiwari et al. (2019) reported that when the dose was less than 4 mg/kg, nCuO improved rice seedling growth (Tiwari et al., 2019). Conversely, dose-dependent toxicity was evident from 5 mg/L to 2000 mg/L, even with a 24 h exposure; Cu ions released and collected from nCuO suspension showed no overt toxicity when re-applied to the same plants (Wang et al., 2015; Yang et al., 2015). These dose-dependent and particle type-specific toxicity align with the current results.

### 2.3.3 Protein, sugar, and starch in grains

Across all the treatments, there was minimal impact on grain protein content. Only exposure to 75 mg/kg of bCuO significantly decreased cultivated grain protein by 57.7% with respect to the controls, suggesting greater tolerance of weedy rice against CuO-induced stress (p ≤ 0.05). This may be related to the lower Cu concentration in the weedy and cultivated rice grains at harvest (Fig. 2.2). Interestingly, grain sugar and starch content varied significantly with rice cultivar (Fig. 2.5). Specifically, after the exposure to nano and bulk CuO at 75 mg/kg, cultivated rice grain accumulated starch grain at levels 41.6% and 92.0% greater than the controls, respectively; whereas, weedy rice had decreases in starch by 70.6% and 74.5% at 150 mg/kg, respectively. A similar trend was evident in grain sugar content. In plants treated with 150 mg nanoscale CuO/kg and 75 mg bulk CuO/kg, a significantly greater sugar content was found in cultivated rice grain as compared to the weedy rice (p ≤ 0.05). Treatment with CuSO₄ had no
impact on grain protein, starch, and sugar content.

2.3.4 Grain nutrient element content

The concentrations of nutritional elements accumulated in grains and spikes of two rice varieties are shown in Table 2.1. Importantly, Cu-based exposure only decreased the nutritional content of elements in grains of weedy rice; no decreases were found in cultivated rice. In addition, bulk CuO induced greater decreases in nutrient content than did nanoscale CuO in weedy rice.

Potassium (K), phosphorus (P), and sulfur (S). The accumulation of K in the grains of cultivated rice was not affected by treatment; however, weedy rice treated with 150 mg/kg of nCuO, 75-150 mg/kg of bCuO, or 300 mg/kg of ionic CuSO$_4$ had a decreased K content of 47.4%, 52.9-77.1%, and 55.2%, respectively, compared with the controls ($p \leq 0.05$). The greatest deficit was observed with bulk CuO treatment at 150 mg/kg (4,456 mg/kg DW), which is significantly lower than the corresponding values with nanoscale CuO (10,237 mg/kg) and the controls (19,461 mg/kg) ($p \leq 0.1$). This pattern is positively correlated with grain Cu uptake of weedy rice, where more Cu was translocated to grains with nanoscale CuO than bulk CuO ($p \leq 0.05$). Previous studies have shown that nanoscale CuO could induce the overproduction of citrate and reactive oxidative species (Wang et al., 2020), which can damage the lipid bilayer and result in K$^+$ efflux (Demidchik, 2015). Since rice was not significantly affected by CuSO$_4$ at $< 300$ mg/kg, our result suggested a particule-specific effect of nanoscale and bulk CuO on grain K accumulation. Previous studies has shown that plant mineral nutrient accumulation was differently affected according to their varieties (Wang et al., 2019). A cultivar-dependent decrease of K uptake was found between green and rosie bok choy (Brassica rapa subsp. chinensis) after exposure to nanoscale/bulk CuO (Deng et al., 2020a). Specifically, the K content was reduced in the leaves of the Rosie variety but not in the Green cultivar. This was correlated primarily to differences in anthocyanin content and pH value.
in leaves (Deng et al., 2020b; Tan et al., 2018). In addition, reduced K content was also found in nanoscale/bulk Cu(OH)$_2$ treated alfalfa (Medicago sativa) seedlings (Cota-Ruiz et al., 2018).

The content of grain P and S was similar to K. No changes were found in the cultivated rice, again highlighting a variety-dependent effect. In the weedy cultivar, the concentrations of both P and S were decreased only by bulk CuO at 75 and 150 mg/kg; the nanoscale and ionic Cu had no such impact (p ≤ 0.05). Specifically, the decreases were 43.0-39.4% and 34.4-32.5% for P and S, respectively, compared with the controls. Similar findings were reported by Rawat et al. (2018), who treated bell pepper (*Capsicum annum* L.) with nCuO, bCuO, and CuCl2 during a full life cycle study. Only bCuO caused lower P absorption in roots (36%) as compared to the controls. Conversely, Hong et al. (2015) reported that P uptake in the roots of lettuce and alfalfa was reduced by >50% by all Cu compounds (nCu, bulk Cu, nCuO, bulk CuO, Cu(OH)$_2$ (CuPRO 2005, Kocide 3000), and CuCl2) at all concentrations (5, 10, and 20 mg/L) (Hong et al., 2015). Although released Cu$^{2+}$ may interact with S and phosphate ions to form complexed precipitates such as CuS, Cu(HPO$_4$)$_2$, or Cu$_3$(PO$_4$)$_2$, no changes were caused by exposure to ionic CuSO$_4$. Sulfur is uptaken by Sultr 1;1 and Sultr 1;2 transporters (Gigolashvili and Kopriva, 2014) and is involved in the synthesis of many metal-binding polypeptides, such as metallothionein and phytochelatins (presented in the form of thiol (–SH)), and are also involved in plant antioxidant capacities. However, further studies are needed to understand the rice variety-dependent responses.

Magnesium (Mg), Calcium (Ca), Zinc (Zn) and iron (Fe). In the weedy rice, grain Mg content was decreased by 34.2% and 77.2% upon exposure to nanoscale and bulk CuO, respectively, as compared with controls; but ionic Cu had no impact on the weedy grain Mg content and again, no significant changes were found in the cultivated cultivar (p ≤ 0.05). Interestingly, decreases in Ca and Zn content were evident in the ion Cu treated weedy rice grains. Again, a
cultivar- and Cu-dependent effect was observed. Upon exposure to nanoscale CuO, the Mg content was decreased from 5118.4 mg/kg (control) to 3794.5 mg/kg; the reduction induced by bulk CuO was more significant at 1312.9 mg/kg. Nearly the same trend was detected in the uptake of Ca and Zn. Bulk CuO caused significantly lower Ca and Zn in weedy rice grains by 95.8% and 82.6%, with respect to nanoscale CuO and the controls (p ≤ 0.05). Similarly, Alaoui-Sossé et al. (2004) reported a reduction of Ca2+ in plants treated by ionic copper in sand-grown cucumber (Alaoui-Sossé et al., 2004); Trujillo-Reyes et al. (2014) reported similar decreases in hydroponically grown lettuce (Trujillo-Reyes et al., 2014). Bulk CuO possesses a less negative zeta potential than does the nanoscale CuO; the values are -45 mV and -20 mV for bulk and nano CuO, respectively, at pH 7 (Deng et al., 2020a). Given the bulk Cu has greater ion dissolution (Rawat et al., 2018b), bulk Cu may interact to a greater extent with Mg, Ca and Zn ions in soil, forming more insoluble sulfides with decreased bioavailability. In addition, with a much higher surface/volume ratio and more translocation amount to the grains, nanoscale CuO may retain positively charged metal ions when uptaken by the plants; thereby, minimizing the negative impact on grain nutritional element accumulation. This potential explanation is also supported by our data of Cu content in grains.

The accumulation of Fe in the cultivated rice was increased by exposure to 75 and 150 mg/kg of nanoscale CuO; importantly, levels went from 7.9 mg/kg in control to 14.9 and 12.1 mg/kg, respectively (p ≤ 0.05). This suggests the potential use of nCuO as nanofertilizer to increase the Fe content in crops. No response was observed in the corresponding bulk and ion Cu treatments, or the weedy rice cultivar. Conversely, bulk CuO at 150 mg/kg reduced grain Fe content by 69.1% in weedy rice. The comparison between nanoscale CuO, bulk CuO and ionic Cu treatments demonstrate that the properties of different CuO forms play a vital role in the uptake of Fe in cultivated rice grains. Deák et al. reported that the stress caused by nanoparticle exposure
increased the production of ferritin in tobacco vegetative tissues, an intracellular protein with a high affinity to bind and store Fe (Deák et al., 1999). This nanoscale-specific effect of CuO was also reported by Wang et al. (2020) in green onion (*Allium fistulosum*) plants. In that study, significantly more Fe was detected in onion roots exposed to nanoscale CuO (at 75 and 150 mg/kg) than the controls, as well as bulk CuO and ion CuSO₄ treated plants.

### 2.3.5 Spike nutrient element content

K, P, and S. As shown in Table 2.1, the nutrient element accumulation in rice spikes displayed the nearly opposite trend of that in the grains, and levels were statistically different between the nanoscale and bulk CuO treated plants. The concentrations of K, P, and S in the spikes of both cultivated and weedy rice were all significantly enhanced by bulk CuO at 75 and/or 150 mg/kg but no such change was found in nanoscale CuO treatment. Ionic CuSO₄ increased spike K and S content in a concentration-dependent manner but only in weedy rice. Rawat et al. (2018b) reported that soil amended with CuCl₂ enhanced S content in bell pepper by 71% in the roots and by 40% in leaves (Rawat et al., 2018b). In another study, all Cu compounds (nCu, bulk Cu, nCuO, bulk CuO, and CuCl₂) caused higher S accumulation in lettuce roots, alfalfa roots, and shoots (Hong et al., 2015). This synergistic effect between Cu and S uptake may be due to the formation of copper sulfate or other related organic complexes (Flemming and Trevors, 1989).

Since nanoscale and bulk CuO led to a similar Cu translocation to rice spikes, there was no clear relationship between spike Cu and K, P, and S content. According to a previous microscopy study, bok choy plants treated nanoscale CuO had a greater portion of Cu in the leaf parenchyma rather than midrib as compared with those treated by bulk CuO (Deng et al., 2020a). This particle size and plant organ-dependent results align with our current findings. In our study, bulk CuO tended to result in K, P, and S retention within the rice spikes, rather than enabling delivery to the
grains. These findings correspond with the Cu translocation data. As shown in Fig. 2.2, Cu content in spikes treated with bulk CuO was significantly higher than controls, while no further enhanced translocation of Cu to grains was evident. Similarly, in nanoscale CuO treated plants, greater Cu translocation to the grains promoted the delivery of P and S to grains, and thus, no overt accumulation of P and S was found in spikes. This “phytostabilization effect” which keeps nutrient elements staying in grain rather than moving to the spike, was reported previously. In green onion (Allium fistulosum) exposed to nanoscale/bulk CuO and CuSO₄ at the same concentration as the current study, alteration of element content was greater in plant bulbs than in roots, although a higher Cu content was observed in roots (Wang et al., 2020).

Mg, Ca, Fe and Zn. Mg accumulation was increased in the spikes of weedy rice across nearly all the treatments, but no such effects were noted in cultivated rice. Bulk CuO at 75 and 150 mg/kg resulted in the highest Mg values of 12,440 and 14,293 mg/kg, respectively, compared with the controls at 3745.6 mg/kg (p ≤ 0.05). The same trend was found with Ca content. Compared with controls, nanoscale CuO increased Mg and Ca content in weedy rice spikes by 218.1 % and 302.6 %, respectively (p ≤ 0.05). The spike Fe content was increased by bulk CuO exposure in both cultivated and weedy rice at 75 and 150 mg/kg by 191.7 % and 364.7 %, respectively, compared with each control (p ≤ 0.05). Nanoscale CuO at 75 and 150 mg/kg increased Fe and Zn accumulation (~30.2 and ~190.2 mg/kg) only in weedy rice spikes with respect to control (12.9 and 93.3 mg/kg), while declined Zn content in cultivated rice (p ≤ 0.05). Importantly, the findings clearly illustrate that nearly all the changes (mainly positive) were only observed in the weedy rice cultivar. Compared with cultivated rice, weedy rice showed a preference to store nutritional elements (except Fe) in spikes and to inhibit further translocation to the grains, particularly with bulk CuO treatment.
2.3.6 Auxin associated gene expression

As shown in Fig. 2.6, auxin associated gene expression levels were measured in grains from both weedy and cultivated rice. No significant alteration was found in the expression of Cu/ZnSOD related gene. In cultivated rice grain, auxin associated gene expression was significantly up-regulated up to 5.23- and 7.90-fold in response to nCuO and bCuO exposures at 75-150 mg/kg, respectively, compared with control; in weedy rice, the corresponding upregulation was 1.60- 1.40- and 1.98-fold, respectively (p ≤ 0.05). This result aligns with a previous study of wheat (*Triticum aestivum* L.), where 15.6 μM nCu resulted in elevated levels of genes related to auxin efflux transmembrane transport in plant roots (Zhang et al., 2018). Auxin, a major plant hormone and signaling molecule, not only regulates plant growth but also plays a significant role when plants are under abiotic stress by activating the salicylic acid signaling pathway that enables crosstalk with other hormones such as gibberellins and cytokinins (Verma et al., 2016). Auxins regulate the morphology and architecture of plant roots through interaction with ethylene, which enhances plant tolerance of abiotic stresses such as drought, salinity, flooding, and nutrient deficiency (Kohli et al., 2013; Negi et al., 2010). Importantly, auxins are associated with plant responses to metal stress (Singh et al., 2021) and stimulate the transcription of primary auxin response genes related to oxidative stress (Huang et al., 2008). Hence, upregulation of auxin associated gene levels in rice grains may serve to alleviate stress-induced by Cu-based compounds.

2.4. Conclusions

This study found cultivar-dependent responses upon exposure to Cu-based materials in grain PFG, GAW, sugar and starch content, and grain micro- and macro-element accumulation. In fact, iron concentrations are increased by nCuO in rice, and this suggests the potential use of nCuO as
nanofertilizer. Moreover, particle size-dependent effects between nanoscale and bulk CuO treatments were clearly evident in the results of grain Cu accumulation, grain yield, and content of nutritional elements. Overall, bCuO exerted greater toxicity or negative impacts than nCuO did. The Fe concentration in cultivated rice grain and the expression of auxin associated gene in the grains of both varieties were increased by nCuO. Importantly, the magnitude of adverse impact on grain nutritional accumulation was greater in wild rice than in the cultivated variety. These findings increase the understanding of nanoparticle uptake and translocation mechanisms in plant edible tissues and provide valuable information for applying nanomaterials in delivery systems for nano-enabled fertilizers or crop protection materials. Further work is required to understand the cultivar and particle size-specific responses of rice to CuO to optimize this potential approach in nano-enabled agriculture.
Fig. 2.2
Fig. 2.3
Fig. 2.4
Fig. 2.5

(A) Normalized grain sugar content

(B) Normalized grain starch content

Cultivated Rice

Weedy Rice

Legend:
- 0
- 75
- 150
- 300
- 600

Data points with different letters indicate significant differences.
Fig. 2.6
<table>
<thead>
<tr>
<th>Rice organ</th>
<th>Element</th>
<th>Cultivar</th>
<th>Treatment</th>
<th>Concentration (mg/kg)</th>
<th>Mean (mg/kg)</th>
<th>Stand error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grain</td>
<td>K</td>
<td>Weedy</td>
<td>Control</td>
<td>0</td>
<td>19461.39</td>
<td>2340.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>nCuO</td>
<td>150</td>
<td>10236.65</td>
<td>1807.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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Chapter 3: Soil and foliar exposure of CuO nanoparticles to Soybean (Glycine max): Coating and particle size-dependent Cu accumulation

3.1 Introduction

It is now clear that increasing population growth will exert tremendous pressure on global agriculture and challenge efforts to achieve food security. The year 2020 was reported as the worst year of famine in the past 60 years, with nearly 250 million people experiencing hunger; this value is nearly double the levels in 2019. To accommodate the growing population, most estimates indicate that agricultural output will have to increase by 60-70% by 2050 (Lowry et al., 2019). In addition, declining arable land and a changing climate will further challenge agriculture production, likely requiring the growth of crops on more marginal lands and under more extreme conditions. Another issue of concern is the current lack of sustainability of many current agricultural practices. For example, the efficiency of agrochemical delivery is often less than 30%, and in some cases, is less than 5% (Kah et al., 2019). Growers must overapply agrochemicals to achieve the desired yield but this overapplication not only wastes energy and water but also causes significant environmental damage. In response to these many challenges, novel and sustainable food production strategies are urgently needed to combat rising global food insecurity (Pawlak and Kołodziejczak, 2020). A number of nano-enabled agricultural approaches have shown significant promise as strategies to enhance agrochemical efficacy and crop yield. For example, nanoscale fertilizers such as N, P, K, Fe, Mn, Zn, Cu and their oxides have shown better release and targeted delivery efficiency than conventional agricultural fertilizers (Dhoke et al., 2011; Pradhan et al., 2013; Rop et al., 2019; Wang et al., 2021a; Yatim et al., 2018). Also, compared with conventional pesticides, metal-based nanoscale pesticides such as Cu, Ag, and SiO2 show better efficiency at
high dose in controlling plant diseases and insect pests (Dong et al., 2021; Dzimitrowicz et al., 2018; Elmer et al., 2021; Sun et al., 2019). However, targeted research is needed on developing strategies that can be readily scaled up and that can achieve both regulatory and consumer acceptance.

A range of Cu-based nanomaterials have demonstrated significant potential for application in a range of industries. Copper oxide (CuO) nanoparticles (NPs) are one of the most common Cu-based nanomaterials, and are widely used in various commercially as gas sensors, catalysts, semiconductors, and photovoltaic cells (Croteau et al., 2014) (Abramova et al., 2013). Although dose is obviously critical, CuO NPs can be rather toxic to a range of biota, particularly microbial species. These particles may dissolve or biotransform during absorption and translocation in organisms such as plants, making predicting and understanding nanoscale toxicity a challenge (Arif et al., 2018). Conversely, at lower doses CuO has also been investigated in agriculture as a crop protection strategy (Elemike et al., 2019) (White and Gardea-Torresdey, 2018). Additionally, after exposure to CuO NPs or other metal-based NPs, adverse effects of plants have been widely reported (Pelegrino et al., 2021; Peng Yuan et al., 2021; Rajput et al., 2019; Wang et al., 2020). For instance, CuO NPs inhibited the growth of seedlings of different Arabidopsis ecotypes (Wang et al., 2016) and reduced Bok choy leaf biomass and chlorophyll content (Deng et al., 2020). The morphology of the carrot taproot exposed to TiO2 NPs changes, and the split phenomenon was discovered (Wang et al., 2021c). Ag NPs also led to the production and accumulation of ROS in rice (Wang et al., 2021d). In addition, metal nanoparticles are usually coated with organic compounds to increase the properties of the nanoparticles, such as dispersion and stability in various media. By changing the physicochemical properties of nanoparticles, this surface modification may also change their toxicity (Iannone et al., 2021) (Dimkpa, 2018). For instance,
soil applications of citric acid-modified CeO2 NPs increased the shoot length of tomato (Solanum lycopersicum L.) plants at 500 mg/kg (Barrios et al., 2016). After surface modification (hydrophilic and hydrophobic), TiO2 NPs significantly improved the biomass of carrot plants at 400 mg/kg and 100 mg/kg (Wang et al., 2021b). Moreover, triethoxycaprylylsilane coated ZnO NPs increased bean plant's root and leaf length (Medina-Velo et al., 2017). However, surface modification does not necessarily reduce the toxicity of metal nanomaterials. For example, polymer-coated copper oxide nanoparticles exhibited 10 times stronger toxicity in Lemna gibba plants than bare CuO NPs (Perreault et al., 2014). The mechanism involved in this kind of effect is not fully understood, and therefore, the possible impact of surface modification is unpredictable. Moreover, a technique used to study these effects has attracted considerable attention, two-photon microscopy, because of its deeper tissue penetration and strong signal background suppression. The advantage of this microscopy is that the internal structure can be observed by fluorescence without extensive sample preparation. For instance, two-photon microscopy images show that the pericytes and cortex of the roots of sweetpotato exposed to CuO NPs have higher fluorescence compared with bulk CuO (Bonilla-Bird et al., 2018). It has also been used with graphene quantum dots gold nanoparticle conjugates to sense and image endogenous biological cyanide, thereby realizing the sensing and imaging of CN- in different types of plant tissues (Wang et al., 2015).

Soybean (Glycine max) was chosen as a model crop in this study because it is a widely cultivated plant, serves the important ecosystem service of symbiotic nitrogen fixation, and is an excellent source of plant-based protein in the human diet. CuO NPs were have demonstrated significant potential in a range of fertilizer and crop protection studies. However, citric acid-coated CuO NPs (CuO-CA NPs) have much higher stability in aqueous media and lower overall solubility as compared to bare CuO NPs. As such, these two nanomaterials were directly compared in the
current study. Here, soybean plants were grown in a growth chamber for 65 days, and three types of copper-based materials (Cu(NO3)2, CuO-CA NPs, and commercial CuO NPs) were applied by either soil or foliar exposure. The measured endpoints included wet and dry biomass, photosynthetic parameters, fruit total protein content, and nutrient content in the different plant tissues. Additionally, two-photon microscopy was used to study the biodistribution and translocation patterns of CuO and CuO-CA NPs in the plant tissues. This study will increase our understanding of the role of material chemistry in optimizing beneficial effects of nano-enabled agriculture while simultaneously minimizing risk and toxicity.

3.2. Materials and Methods

3.2.1 Material preparation and characterization

Particulate CuO NPs and ionic Cu (Cu(NO3)2) compounds were received from The University of California Center for Environmental Implications of Nanotechnology (UC CEIN). Their physicochemical properties have been documented previously (Rawat et al., 2018). CuO-CA NPs were synthesized according to the method of Mallakpour et al (Mallakpour et al., 2015). Briefly, 1 g of CuO NPs were suspended in 100 mL of Millipore water (18MΩ) (MW): ethanol solution (8:2 v/v) and were sonicated for 1 h. Then, 7.2 g of citric acid was dissolved into another 100mL of MW: ethanol solution (8:2 v/v). The CuO NPs solution was then mixed with citric acid solution in a round-bottomed flask, the pH adjusted to 7 with 6M NaOH solution, and the solution was refluxed for 3 hours. Once coated, the CuO-CA NPs were isolated by centrifugation at 4000 RPM for 5 min, washed, and oven dried (70 °C) for 24 hours. Thereafter, suspensions of the Cu-based compounds (CuO NPS, CuO-CA NPs, and Cu(NO3)2) were prepared in MW water at 75 and 300 mg/kg, and sonicated at 25 °C for 30 min before use. CuO NPs and CuO-CA NPs were
compared to understand the effect of surface coating, while the ionic compound was used to
differentiate between the nanoscale and ionic effects.

CuO-CA NPs were characterized by powder X-ray diffraction (Panalytical Empyrean 2,
UK). The geometry and space group of the CuO NPs were found to be monoclinic and C2/2,
respectively. Additionally, the crystallite sizes of the materials were calculated using Scherrer’s
equation. To ensure citric acid functionalization, FT-IR (Agilent Technologies, USA) was used to
investigate the functional groups. Moreover, the properties of the various CuO NPs in the solution
(300 mg/L) were investigated by a Zetasizer (Malvern, UK) to determine the hydrodynamic size
and zeta-potential at pH 7. For both measurements, the CuO suspensions were sonicated at 25 °C
for 30 min to avoid aggregation.

3.2.2 Experimental design

Plastic pots (12.5 cm diameter × 14 cm height) were washed with MW and filled with 3 kg
of commercial potting mix soil (Miracle-Gro® Potting Mix), purchased from a local market in El
Paso, TX. The characteristics of the potting soil have been previously reported (Cota-Ruiz et al.,
2020). The soybean seeds were germinated in a starter germination kit (9GreenBox), and after 15
days, the seedling was transplanted into the pots. After sowing, seedlings and plants were grown
in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) under conditions of
25 ± 5 °C day/night temperature, 60 ± 10 % relative humidity, and 12 h photoperiod. Plants were
regularly watered (MW) to maintain approximate 60 % of their field capacity in the soil (every ~2
days), and no additional fertilizer was used. The pots were laid out in a completely random design.

For the soil exposure treatment, the soil was separately amended with CuO NPs, CuO-CA
NPs, and Cu(NO3)2 suspension/solution to give final Cu concentrations of 75 and 300 mg/kg per
pot. These concentrations were selected based on previous studies that indicate these levels are
encountered in field soils (Yang et al., 2017). After the suspension/solution was added into the 3 kg of soil, the soil was manually mixed for more than 30 min until homogeneous. For the control plants, only MW was added. For the foliar treatment, suspensions/solutions were added into different commercial spray bottles and sonicated each time before use. The plants were sprayed with 5 mL of the suspensions every 5 days for 3 times (15 mL in total), and the first spray was applied 5 days after transplanting. As a result, 1.125mg (75mg/L) and 4.5mg (300mg/L) of copper were given in each pot, in line with the work of Tan et al. (Tan et al., 2018). The soil surface was covered with plastic wrap prior to application to avoid contamination. Moreover, the plants were transferred to fume hoods before applying each treatment to prevent cross-contamination. Control plants were only sprayed with MW. Five replicates were established for each treatment.

**3.2.3 Plant harvest**

Plants were harvested at 50 days after transplanting. Samples of roots, stem and leaves were acquired and thoroughly cleaned 3 times with MW to remove the soil and nanomaterials adhered on the surface prior to further analysis. Before harvesting, the average chlorophyll content of five randomly selected leaves from each plant was evaluated by a handheld single-photon avalanche diode chlorophyll meter (SPAD, Minolta Camera, Japan). After harvesting, fresh samples were weighed and then oven-dried for 3 days at 70 °C to determine the dry mass.

**3.2.4 Nutrient analysis**

To determine the elemental content in tissues, dry samples were ground in a mortar with liquid nitrogen, and 0.2 g was weighed into 50 mL digestion tubes, followed by the addition of 2.5 mL trace pure nitric acid to each sample. The samples were then digested in a digiPrep hot block (SCP Science, Quebec, Canada) for 45 min at 115°C. The digested tubes were brought to 45mL
with MW and the concentration of select macro- and micronutrients (S, P, K, Mn, Mg, Ca, Fe, Zn, Al, Ni, and Cu) were determined using an inductively coupled plasma-optical emission spectrometer (ICP-OES, Perkin-Elmer Optima 4300 DV; Shelton, CT). For quality control, a blank sample and a Cu NPs spiked sample of known content were analyzed every 30 samples. A standards reference material (NIST 2709a, Gaithersburg, MD) was also tested as part of our QA/QC.

3.2.5 Total Protein Extraction and Analysis

Two hundred mg fresh tissue was mixed with 1.5 mL of phosphate buffer (pH 7.4) and 20 mg of polyvinylpyrrolidone in a 2 mL tube. The tissues were centrifuged at 12,000 RPM, and the extract was isolated and used for quantification. The quantification of total proteins was determined using Bradford’s colorimetric technique (Bradford, 1976). Specifically, 5 μL of the sample mixture and 250 μL of Bradford reagent were added in a microplate well. They were incubated at room temperature for 10 min and then read on a microplate reader (Multiskan SkyHigh Microplate Spectrophotometer; Thermo Fisher Scientific, Fluoroskan, FL) at 595 nm. Calibration curves were constructed using the bovine serum albumin as a standard. The total proteins were expressed in μg/g fresh sample.

3.2.6 Copper release in water

Copper dissolution in suspension was determined for CuO NPs and CuO-CA NPs species. Cu(NO3)2 was not tested as it is completely soluble in water. Specifically, 300 mg/L of either CuO or CuO-CA NPs were prepared in 50 mL conical vials. Suspensions were shaken for 2, 4, 8, 24, 96, 192, and 384 hours (h). The samples were stationed upright for 2 h after shaking to allow settling, and centrifuged at 7000 rpm for 15 min (Sorvall, Legend XR1 centrifuge, Thermo Fisher
Scientific, Waltham, MA, USA). Afterward, 5 mL of supernatant was decanted and filtered to leave only dissolved chemicals in the solution. The final solution was analyzed by ICP-OES to determine dissolved Cu over time.

3.2.7 Sample preparation for Two-photon microscopy images

Root, stem, and leaf samples of plants exposed to CuO NPs, CuO-CA NPs, and Cu(NO3)2 at 300 mg/kg (soil and foliar) were harvested at day 50. Each sample was sectioned to a thickness of approximately 200 μm with microtone (Leica CM1850; Buffalo Grove, IL) and mounted in a water-immersion objective lens (Olympus LUM Plan FLN). The light source used was a mode-locked femtosecond titanium: Sapphire laser (Spectra-Physics, Mai-Tai HP). The pulse width is about 100 fs, and the repetition rate was 80 MHz. To obtain two-photon excitation, the wavelength was set at 720 nm light, and 120 mW laser power was transmitted at the sample point. The fluorescence signal of the sample was bent with a 665 nm long-range dichroic mirror. LDBS (long-pass dichroic beam splitter) was used to distribute blue and green/red fluorescence signals. Different signals pass through the band-pass filter, which are 417-477nm for blue, 500-550nm for green and 570-616nm for red. Final detection is by photomultiplier tube (PMT). The output of these three PMT signals is sent to red/green/blue channels of the image acquisition card installed on the computer. The two-dimensional images in the x–y plane is obtained by a self-developed software program. The imaging speed of this was 30 frames/sec and the final static image had around 50 FPS. In addition, some images will display red artifacts when processed by the image acquisition card, such as stripes on the image; therefore, artifacts were eliminated by performing minimal image processing using the ImageJ 1.51j8 software (National Institute of Health, USA)(Acosta et al., 2014).

3.2.8 Statistical analysis
All agronomic, elemental, and dissolution data were analyzed with the SPSS program 26 (SPSS, Chicago, IL, USA). A one-way ANOVA and Tukey HSD multiple comparison test was used to compare the mean values of the control group and the treatment group. Results are reported as mean ± standard error, and significant differences were determined at P ≤ 0.05.

3.3. Results and discussions

3.3.1 Characterization of NPs

The physical characterization data of both NPs are shown in Table 1. CuO-CA has much lower zeta-potential than does CuO. The reason may be that citric acid binds CuO, which causes the NPs to be less susceptible to agglomeration in a water system. The particle size of CuO NPs is smaller than that of CuO-CA NPs, although the hydrodynamic size indicates the opposite. This is the result of the citric acid coating changing the charge of the electric double layer on the CuO surface, yielding a smaller hydrodynamic size. The FT-IR (Fig S1) data showed that CuO-CA contains C – O stretching and C = O stretching that correspond to citric acid at ~1150 and ~1600 wavenumbers (cm⁻¹). This data confirms successful functionalization of the CuO.

The dissolution results indicate that CuO-CA NPs release greater amounts of Cu in aqueous solution than CuO NPs. At 2 h, CuO-CA and CuO NPs released 0.830 and 0.015 mg/L, respectively (Fig S2). By 192 h, CuO released 0.037 mg/L Cu, whereas CuO-CA released 2.05 mg/L. Although the overall amount dissolved from the coated nanoparticles is much greater, the pattern of release for the two materials is similar. The reason behind this may be due to the acidic nature of the citric acid coating. As the citric acid is dissolves in the aqueous solution, this will decrease the pH and encourage greater metal oxide dissolution (Yokoyama et al., 2018).
3.3.2 Cu is accumulated in different plant organs in an applied method dependent manner

The amount of Cu in leaf, stem, and root tissues are shown in Fig 1. As shown in Fig. 1a, both nanomaterials significantly increase the content of Cu in the roots, particularly with the soil application of 300 mg/kg; specifically, the CuO and CuO-CA NPs caused an increase from 5.9 mg/kg (control) to 45.7 mg/kg and 208.3 mg/kg, respectively. Conversely, the root Cu content upon foliar application was 9.5 mg/kg and 9.3 mg/kg. Not surprisingly, the Cu content in the roots with the soil treatment is significantly higher than that of foliar treatment (p ≤ 0.05). This is a function of the roots being in direct contact with nanomaterials in the soil, maximizing the likelihood of root absorption. Similarly, Salehi et.al found that NP accumulation by plants was a direct function of the amount and mode of application (Salehi et al., 2018). Interestingly, in foliar treatment, only the 300 mg/L of each nanoparticle lead to root content greater than the control (6.0 mg/kg); the values for CuO and CuO-CA NPs root Cu content were 10.5 mg/kg and 10.4 mg/kg, respectively. The low 75 mg/L dose of each nanomaterial did not lead to enough absorption and phloem loading of Cu to induce a significant difference in the root Cu content. Interestingly, regardless exposure methods, the low concentration of ionic Cu had no impact on root Cu content. However, at the high concentration of ionic Cu, the root Cu content via the soil treatment was significantly increased to 89.3 mg/kg, whereas in the foliar treatment, the Cu content in the root was significantly reduced (3.1 mg/kg). This decrease seems to be a function of the foliar ionic exposure leading to Cu enrichment in other plant tissues. In this experiment, we found that Cu is more enriched in the pod.

The leaf copper content after exposure via soil and foliar application is shown in Fig 1c. In the case of soil application, the high concentration of CuO NPs treatment (10.4 mg/kg) was significantly greater than the control (5.4 mg/kg)(p ≤ 0.05). In addition, the CuO-CA NPs at all
concentrations increased the content of Cu in the leaves (11.0 mg/kg, 15.8 mg/kg) compared to control. By comparison, CuO-CA is easier for plant leaves to absorb Cu than CuO NPs. This may be due to the coating increased biocompatible effect on NPs (Barrios et al., 2016). In the foliar treatment, all concentrations of both CuO NPs (19.0 mg/kg, 52.6 mg/kg) and CuO-CA NPs (49.2 mg/kg, 131.6 mg/kg) have increased the Cu content in plant leaves, compared to control (5.4 mg/kg). This is because the stomata through plant leaves are one of the main ways for nanomaterials to enter the plant body, thus, affecting the element content in the leaves (Jie Hong et al., 2021). In addition, all Cu ion treatments in both exposure methods also significantly increase the content of Cu in the leaves by 12.6 mg/kg and 63.0 mg/kg.

As shown in Fig 1b, all soil treatments significantly increased the Cu content in the stem; this was particularly evident with 300 mg/kg of either CuO NPs, CuO-CA NPs or ionic Cu which increased the stem copper content by 5.1 times, 5.2 times and 6.0 times, respectively, compared to control. For the foliar application, all treatments also significantly increased the Cu content in the stem, excluding the 75 mg/L ionic treatment. Again, 300 mg/kg, CuO NPs, CuO CA NPs, and ionic Cu, increased the stem Cu by 3.7 times, 14.5 times and 4.7 times, compared to control.

The Cu content of the edible tissues (pods and seeds) are shown in Fig 2. In the soil application, the 300 mg/kg of CuO NPs (14.2 mg/kg) and all concentrations of CuO-CA NPs (14.6 mg/kg, 15.2 mg/kg) significantly increased the Cu content in the pods. Both Cu ion treatment (12.2 mg/kg, 15.4 mg/kg) also significantly increased the Cu content, compared to control (7.1 mg/kg)(p ≤ 0.05). In the case of foliar treatment, all concentrations of CuO and CuO-CA NPs had no effect on the Cu content in the pods. However, the ionic treatment significantly reduced the Cu content in the pod at 75 mg/L concentrations (2.3 mg/kg). Interestingly, the ionic treatment at 300 mg/L significantly increased Cu content in the pod (13.0 mg/kg). This indicates that at low concentrations, Cu ions
are transferred to the roots of plants and are enriched in the roots, but as the concentration increases, more transport to the pod occurs. This also explains the change in Cu content with the Cu ion treatment in the roots (Fig 1a). As shown in Fig 2b, the Cu element content in the seed also varies significantly with application method. In the soil exposure, 300 mg/L CuO NPs (16.6 mg/kg), both concentration of CuO-CA NPs (17.6 mg/kg, 21.0 mg/kg), and both ionic counterpart treatments (14.7 mg/kg, 18.5 mg/kg) increased the seed Cu content compared with the control (7.7 mg/kg). Conversely, for the foliar method most treatments had no impact on seed Cu; the exception was the high-concentration CuO-CA NPs treatment which significantly reduced the seed Cu content to 3.4 mg/kg. The decrease in the Cu seed content at the same treatment under different exposure methods may occur because the plant stores CuO and CuO-CA NPs in the stem. This shows the impact of application method on in planta Cu distribution. For instance, Wang et al. stated that when plants absorb nanomaterials from the root, transfer to other organs through the xylem often occurs readily, including pod and seed (Wang et al., 2012). When the nanomaterials enter the plant from the leaf surface, transfer to other tissues through the phloem can be more restricted.

This study shows that the Cu compounds can be transferred bidirectionally in the plants. In the soil exposure, all Cu-based materials have an impact on all the plant organs. However, CuO only impacts the edible parts of plants at 300 ppm, whereas 75 ppm has no effect. Conversely, CuO-CA NPs caused significant changes regardless of the concentration. For the foliar method, CuO-CA NPs significantly tended to reduce the Cu content in the seed, except at 300 mg/kg treatment. Importantly, none of the NPs tested caused any changes in the edible parts of the plant. The ionic treatment also had no impact on the seed, although there were some changes induced in the pod.
These findings provide important understanding to the future application of coated nanomaterials as components of pesticides or fertilizers.

3.3.3 Cu is differentially distributed in plants exposed to CuO NPs dependent on NPs’ surface properties

The transfer path of copper-based compounds was observed by two-photon microscopy (Fig 3). When plants were treated with 300 mg/kg CuO or CuO-CA NPs by either exposure methods, Cu was observed in the leaves, stems, and roots. The Cu-based compounds and untreated plant tissue (control) samples were analyzed as controls. The specific detection parameters of these materials were then used to ensure that fluorescent signals found in plants were derived from the materials (Fig. S2). The representative fluorescent spots of Cu based nanoparticles are indicated by arrows in the images.

As shown in Fig 3, plant cells produce their own fluorescence, but signals emitted by plants treated with Cu-based materials are clearly distinguishable. Not surprisingly, for the foliar exposure the fluorescence signal of both nanomaterials is significantly stronger for the leaves (Fig 3a-3d). At the same time, the fluorescence intensity of CuO-CA NPs transferred from roots to leaves in the soil exposure is also higher than that of CuO NPs. This is also consistent with the ICP-OES results and may be due to the biocompatibility of citric acid coating, facilitating greater transport within the soybean plants. In addition, it was observed that all Cu-based materials lead to a clustering of response in the stomata of the leaves, with the exception being CuO-CA NPs by soil exposure. This may be due to the fact that plants often transport or store xenobiotics in this area. However, the functionalization of CuO with CA may increase particle biocompatibility and lead to greater in planta distribution (Iannone et al., 2021).
The fluorescence signals of Cu-based compounds in the treated stems are shown in Fig 4a-4d and again, data are consistent with the previous ICP-OES results. It is clear that the fluorescence signal in the soil exposure is more intense than the foliar treatment. This is likely a result the xylem-based transpiration stream and normal flow of nutrients. Moreover, CuO-CA NPs have a larger particle size and a larger negative zeta potential, which likely leads to greater CuO-CA NPs accumulation in the stem (DalCorso et al., 2014). Similarly, signals of Cu-based compounds were found in all treated root samples (Fig 5a-5d), and fluorescence was more intense for the soil exposure. This is not surprising given the more direct proximity of the root tissue to the exposed soil and the fact the shoot to root transport pathways is more limited for analyte such as Cu.

There is data in the literature suggesting that both foliar and soil exposure can lead to NP presence in and transport through the plant vascular system. In previous studies, CeO2 and CuO NPs were observed to pass from the leaf surface of cucumber (Cucumis sativus) to the root (Hong et al., 2016). Another investigation also mentioned that CuO NPs could be transported from the roots to the aerial parts of rice (Oryza sativa L.) plants (Peng et al., 2015). However, the translocation process of CuO and CuO-CA NPs in soybean plants is still unclear, although the current data clearly suggests coating can significantly influence that in planta movement.

3.3.4 Agronomical parameters

The biomass of plant bean (seed and pod), stem, and leaf tissue under the different treatments are shown in Fig 6. Bean is the main edible part of the soybean plant; thus, it is essential to evaluate the impact of Cu-based compounds on this tissue. Under soil amended treatment (Fig 6a), CuO and CuO-CA NPs treatment at all concentrations and the ionic counterpart at 75 mg/kg had no impact on bean mass relative to the control. At 300 mg/kg, the ionic Cu significantly
increased the bean biomass by 89.8% compared with control. However, under foliar exposure the ionic treatment had no impact on bean mass as compared with the control. Conversely, CuO NPs at 300 mg/kg and CuO-CA NPs at 75 mg/kg significantly increased bean biomass by 70.0% and 69.3%, respectively. The stem and leaf weight were also significantly enhanced by foliar application 300 mg/kg of CuO NPs, 75 mg/kg of CuO-CA NPs, and soil exposure at 75 mg/kg of CuO-CA NPs (Fig 6b,c). The greatest stem and leaf weight increases were with foliar application of CuO-CA NPs at 75 mg/kg, which yielded increases of 74.9% and 84.1%, respectively. From the perspective of promoting plant growth, foliar application of CuO-CA NPs at 75 mg/Kg appears to be very effective at promoting plant growth and fruit yield. Plant height is another metric used to determine the impact of nanomaterials on plants (Fig S4a). In the soil exposure, 75 mg/kg and 300 mg/kg CuO NPs significantly reduced the height by 20.6% and 28.3%, respectively, when compared with control. All other treatments had no impact. In the foliar exposure, none of the treatments impacted plant height.

In previous studies, it has been reported that the impact of metal nanomaterials on crop agronomic parameters depends on many factors, including plant species, growth stage, nanomaterial concentration, exposure method, and surface coating (Cota-Ruiz et al., 2018a). As such, reports in the literature are often mixed. For example, Wang et al. found soil exposure of CuO NPs did not impact green onion fresh weight and height (Wang et al., 2020). However, Rohilla et al. found surface-functionalized (dopamine) CuO NPs enhanced the Vigna radiata germination index and overall enzyme activity (Rohilla et al., 2020). On the other hand, ionic Cu may promote plant growth (Borgatta et al., 2018). These studies align with the findings of the current report. Results often differ significantly as a function of amendment strategy; soil versus foliar application. A previous study showed that foliar sprayed CuO NPs improved lettuce
(Lactuca sativa L) biomass and leaf number. However, no such enhancement effect was evident from soil exposure (Kohatsu et al., 2021). In addition, Su et al reported that surface coating materials can prevent aggregation and clogging in the plant leaf stomata, thereby enhancing the possibility of NPs uptake (Su et al., 2020). In addition, surface modification can enhance the adhesion of NPs on the leaf surface, also increasing the likelihood of uptake (Elmer and White, 2016). This likely explains the increased uptake of Cu derived from CuO-CA NPs during the foliar exposure, leading to beneficial impacts on soybean productivity.

3.3.5 Protein content and chlorophyll content

Total protein content was evaluated for bean tissues as a function of treatment and exposure route (Fig. S4b). For the foliar application, there was no significant effect across all treatments. However, soil exposure to CuO NPs at 300 mg/kg significantly reduced protein by 50.1%. Both the CuO-CA NPs and the ionic counterpart have a dose-dependent effect, but no significant difference was observed compared with control. Particularly for the bean part under investigation, protein is essential as it will determine the growth and fate of the soybean plants. Thus, the results show that the treatment of CuO-CA NPs and ionic Cu compounds do not cause damage to the cellular mechanisms involved in protein synthesis and degradation. However, CuO NPs can induce chemical reactions when exposed to plant cells, including the oxidation of biomolecules (proteins or lipids) or the binding between Cu and proteins or other molecules (Ameh and Sayes, 2019), which can reduce the protein content in plants.

Chlorophyll is a critical photosynthetic pigment and changes in its concentration can significantly impact photosynthesis, affecting sugar production and storage, as well as crop quality and yield. Importantly, chlorophyll content was unaffected across all treatments (Fig S4c). The same result has also been confirmed in previous research (Bakshi and Kumar, 2021).
3.3.6 Element accumulation

Table 2 shows the macro- (K, P, Ca, Mg) and micro- (Mn, Fe, Zn) nutrient profile of the different soybean tissues exposed to either CuO, CuO-CA NPs, or Cu(NO$_3$)$_2$. No significant changes were found for B, As, Mo, and S content as compared with controls in all tissues. No significant changes were found in plant stem and seed for all the element accumulation.

3.3.6.1 Macronutrients- In soil exposure treatments, we found potassium (K) concentrations in the pod and leaf were significantly changed upon treatment. In the pod, the change in K was found to be concentration-dependent with CuO-CA NPs. For instance, 75 mg/L CuO-CA NPs decreased the K content by 23.2% compared with controls. However, at 300 mg/L, CuO-CA NPs increased K concentration by 31.4%. In the leaf, both concentrations of CuO NPs and the 75 mg/L ionic treatment increased the K content by 72.1%, 83.9%, and 81.4%, respectively. Phosphorus concentrations also varied among the different plant tissues under Cu-based nanomaterials applications. In the pod, all the Cu-based treatments significantly decreased P concentration, with CuO NPs causing the greatest decrease at 35.0% and 62.6% for 75 and 300 mg/L. In the leaves, CuO NPs showed no effect in the P content; however, the ionic counterpart at both 75 and 300 ppm and CuO-CA NPs at 300 ppm decreased the P concentration by 38.0%, 41.0%, and 35.7%, respectively. In the root tissues, CuO-CA NPs at 75 mg/L had the most significant effect, decreasing the P concentration by 56.7%. Moreover, the Ca content in plant leaves was significantly increased by all treatments, except for the 300 ppm CuO NPs treatment. The 75 ppm CuO NPs, CuO-CA NPs, and Cu(NO$_3$)$_2$ treatments increased the Ca leaf content by 46.5%, 70.6%, and 41.4%, respectively, while 300 ppm of CuO-CA NPs and Cu(NO$_3$)$_2$ increased the Ca leaf content by 78.9% and 76.6%, respectively. For Mg, only 300 ppm CuO-CA NPs increased the leaf content (18%).
Under foliar treatment, the K concentration varied only in the pods. The K content decreased when exposed to all treatments except CuO NPs. When exposed to 75 ppm CuO NPs, the K concentration increased by 23.5% compared to the control. The P concentration was decreased in several soybean tissues upon treatment. For instance, the pod P content was decreased by all treatments except CuO NPs at 75 ppm. The decreases ranged from 27.1% and 76.6%. In the leaf, where 75 ppm CuO NPs and 300 ppm CuO-CA NPs increased P concentration by 60.7% and 36.7%, respectively. However, 300 ppm Cu(NO$_3$)$_2$ decreased P concentration in leaves by 59.2%. Similar results were found in roots, P decreased with 75 and 300 ppm CuO-CA NPs and 75 ppm Cu(NO$_3$)$_2$ by 26.1%, 61.1%, and 38.4%, respectively. On the other hand, the Ca content was significantly increased by all treatments. CuO NPs increased the Ca content by 60.7% and 62.7% at 75 and 300 mg/L, as compared with control. The 300 ppm CuO-CA NPs and 75ppm Cu(NO$_3$)$_2$ treatments also increased the Ca element content by 58.4% and 48.6%, compared with control. Moreover, the Mg concentration in the leaves was significantly increased by 23.5% and 30.2% when exposed to 300 mg/L CuO NPs or CuO-CA NPs.

The K content in each plant organ increased when exposed to CuO NPs, but it shows a dosage-dependent response for CuO-CA NPs. Similar results were reported in Arabidopsis and Alfalfa by Cota-Ruiz et al. and Murphy et al. Here, the authors report that excessive Cu$^{2+}$ can lead to an increase in citrate, which can cause toxicity. To prevent the accumulation of citrate, the cells exhibit citrate efflux, which is accompanied by K$^+$ efflux (Cota-Ruiz et al., 2018b; Murphy et al., 1999). The previous literature also reports reduction in P content with Cu amendment. This may be due to soil negativity that can force CuO to move to the root, which can physically prevent the absorption of some elements. In addition, Cu can be compounded with phosphate (H$_2$PO$_4^-$, HPO$_4^{2-}$), making P unavailable for plants (Ait Ali et al., 2002; Hong et al., 2015; Rawat et al., 2018;
Safaya, 1976). This may also explains the enrichment of P on the leaf surface in the foliar method; the content of Ca and Mg in the leaves was increased by Cu treatments. According to previous reports, Cu tends to replace the adsorption sites occupied by Ca$^{2+}$ and Mg$^{2+}$, particularly in organic matter. At high concentrations, Cu may disturb the cell membrane and cause elements such as Mg to flow into plant cells. In addition, the interaction between nanoscale copper oxide and crops also depends on the growth medium and plant species (Ochoa et al., 2017).

**3.3.6.1 Micronutrients** - In the soil exposure, Fe and Zn were significantly changed in the leaves, while Mn was changed significantly in the roots. The Fe content was significantly reduced by 56.2% and 45.6% when treated with CuO NPs and by 51.2% and 21.4% when treated with Cu(NO$_3$)$_2$ at 75 ppm and 300 ppm, respectively. Similarly, the CuO-CA NPs reduced the Fe content by 31.6% at 300 ppm. The Zn concentration was also decreased by 300 ppm of both NPs treatments and both concentrations of Cu(NO$_3$)$_2$ (p $\leq$ 0.05). In the roots, the Mn content was significantly reduced by all concentrations of CuO NPs and the Cu(NO$_3$)$_2$ treatment. CuO NPs at 75 and 300 mg/L reduced the Mn content by 67.8% and 75.6%, respectively. The Cu(NO$_3$)$_2$ treatment reduced Mn content by 70.1% and 91.6% with 75 and 300 ppm, respectively. No significant differences were observed upon CuO-CA treatments.

Under foliar treatments, similar results were found in the leaves. The Fe concentration was reduced by 13.9%, 36.9%, and 33.8% with 75 ppm CuO NPs, 75 ppm Cu(NO$_3$)$_2$, and 300 ppm Cu(NO$_3$)$_2$, respectively, and CuO-CA had no impact on Fe content. Similarly, Cu(NO$_3$)$_2$ at 75 and 300 mg/L decreased Zn content by 60.1% and 89.9%, respectively; however, both CuO and CuO-CA NPs significantly increased Zn content in the leaves. For instance, 75 ppm CuO increased Zn content by 44.5%, and 300 ppm CuO-CA NPs increased Zn concentration by 39.7%.
Lastly, the Mn root content was only affected by the 300 ppm Cu(NO3)2 treatment, which reduced the nutrient content by 79.1% compared with control.

The significant reduction in Fe content is due to the competition between Fe and Cu in transport pathways (Kochian, 2018). Fe also may be compounded into ferritin (an iron storage protein) in response to the oxidative stress induced by Cu treatment. Importantly, iron deficiency has been recognized as a threat to global public health (Finkelstein et al., 2017). Therefore, the fact that CuO-CA NPs did not reduce the Fe content in leaves under the foliar method is noteworthy. The Zn content was altered as a function of Cu type and exposure method. Ionic Cu reduced the concentration of Zn regardless of the application conditions, but NPs behaved differently, with effects being dependent on exposure method and concentration. It has been suggested that Zn2+ shares a transporter with Cu2+, so excess Cu will reduce Zn translocation (Du et al., 2018). In addition, excess Cu will damage the cell membrane at high concentrations, allowing Zn to enter the cell non-selectively, and given that foliar application puts Cu into direct contact with the leaf surface, which creates the conditions for Zn to increase on the plant leaf (Trujillo-Reyes et al., 2014). Importantly, antioxidant proteins can increase or decrease Zn concentration to protect with critical physiological functions in stressed cells. Certain types of SOD (superoxide dismutase) use Zn/Cu as their co-factors, explaining the accumulation of zinc in response to this effect (Alscher et al., 2002; Cota-Ruiz et al., 2018b). The content of Mn is only affected by Cu(NO3)2 in foliar exposure but is also affected by CuO NPs in the soil exposure. As mentioned previously, CuO NPs are affected by the complex conditions in the soil and release Cu2+ ions at the surface of plant roots, thereby affecting the absorption capability of Mn. Because Mn plays an active role as a protein co-factor in glucose metabolism and fatty acid synthesis, these processes may be down-regulated due to Cu ion excess, leading to Mn release (Alscher et al., 2002; Ma et al., 2018).
However, CuO-CA NPs showed better stability in the soil and did not significantly affect the absorption of Mn.

3.4 Conclusion

The findings show that soybean physiological and agronomic response vary with exposure condition, Cu type and Cu concentration. Importantly, the CuO NPs modified by citric acid have a greater growth promoting effect on plants than bare NPs. Also, foliar applied CuO and CuO-CA NPs increased the yield of soybean; whereas the ionic Cu treatment did not. Meanwhile, the CuO-CA NPs at 75 ppm is more beneficial, with increases in yield without disturbing the enrichment of nutrient elements in the seed, whereas some potential phytotoxicity being evident at 300 ppm. The translocation of Cu from leaf to root and from the root to the leaf was described by two-photon microscopy. Agricultural applications of various Cu forms have also been reported in the literature to elicit species-dependent responses; thus, it is crucial to evaluate globally significant crops other than soybean. Moreover, establishing the effects of nano-based Cu treatments on these important crops can be used at optimal concentrations so as to sustainably increase crop yield and foster efforts to meet global food demands.
Figures

**Soil exposure**

**Foliar exposure**

![Graphs showing mg Cu kg dry root sample, mg Cu kg dry stem sample, and mg Cu kg dry leaf sample for different samples: CuO, CuO-CA, Cu(NO3)2, and CuO-CA for soil exposure, and CuO, CuO-CA, Cu(NO3)2, and CuO-CA for foliar exposure.](image)

Fig 3.1

55
Fig 3.2

Soil exposure

Foliar exposure

(a)

mg Cu/kg dry pod sample

(b)

mg Cu/kg dry seed sample

CuO  CuO-CA  Cu(NO3)2

CuO  CuO-CA  Cu(NO3)2

Fig 3.2
Fig 3.3
Fig 3.5
Fig 3.6
### Tables

**Table 3.1**

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Chapter 4: Conclusion

In the first study, none of the Cu-based tested concentrations produced visible signs of toxicity. Two-photon microscopy images demonstrated the Cu uptake in nCuO, and bCuO treated roots. At all concentrations, nCuO and CuSO$_4$ increased leaf allicin compared with control. The antioxidant enzymes were differentially affected by the Cu-based treatments. Although nCuO significantly increased root GPOX and leaf APOX, it reduced CAT in both roots and leaves. Scallion plants exposed to nCuO improved their essential element (Ca, Fe, Mg, Mn, and Ni) contents. Overall, the data suggest that nCuO at concentrations lower than 600 mg/kg may be used as nanofertilizer for green onion production.

In the second and third studies, the content of Ti in plant secondary roots treated with unaged nTiO$_2$ at 400 mg/kg was in the order of hydrophobic > hydrophilic > pristine regarding the respective treatments. The two-photon microscope images suggested an uptake pathway independent of the surface coating chemistry of nTiO$_2$. The growth of taproot was significantly inhibited by all unaged nTiO$_2$ forms. Remarkably, an abnormal increase of taproot splitting was found. The accumulation of nutrient elements (Mg, Mn, and Zn) was decreased in taproots treated with the unaged surface-coated nTiO$_2$. On the other hand, most of these inhibition effects disappeared after nTiO$_2$ aging in soil. The aged nTiO$_2$ with surface coatings improved plant agronomic parameters and nutrient element accumulations. More stimulation effects were shown by aged hydrophilic and hydrophobic-coated nTiO$_2$ than the aged pristine nTiO$_2$. These may be related to the changes in surface charges on nTiO$_2$ after aging. It suggested a potential strategy to alleviate the phytotoxicity of released surface-coated nanoparticles; and moreover, offers a sustainable method to convert them into potential stimulators for the growth of plants.
Taking an overview of the three presented studies, the interaction between NPs and plants depend on nanoparticles type/size, plant varieties, exposed conditions, and NP surface coatings. Results in these studies provided valuable insights into the interaction of nCuO with green onion, and surface-coated nTiO$_2$ (aged and unaged) with carrot plants. The potential use of nCuO and aged nTiO$_2$ as nanofertilizers has been suggested in the agrosystem. Meanwhile, the potential risk of releasing unaged nTiO$_2$ into the environment has been emphasized. More studies at molecular levels and considering the full terrestrial food chain are needed to evaluate the potential effect that plants grown in ENMs-amended soils have on humans.
Supporting material for Chapter 2-3

Figures

Fig. S2.1.

Fig. S2.2.
Fig S3.1
Fig S3.2

This figure shows the copper concentration (ppm) over time (h) for CuO-CA and CuO. The graph indicates a decrease in copper concentration over time for both materials, with CuO-CA showing a slightly higher concentration than CuO at most time points.
Fig S3.3
Fig S3.4
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Chapter 1

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Chapter 2


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Vita

Chaoyi Deng was born on September 29th, 1990 in Hunan province, China. He graduated from No.1 middle school in Changsha in 2009. Afterwards, he earned his Bachelor of Science degree in Environmental Science and dual degree in Business management in University of Science and Technology of China in 2013. In fall 2013, he participated in the Joint Training Laboratory Internship program at National center for Nanoscience and technology. In 2015, Chaoyi began his master’s degree in Chemistry science under the mentorship of Dr. Jorge L. Gardea-Torresdey, of which he holds a 3.94 GPA. In August 2018, Chaoyi start his Ph.D. degree in Environmental Science and Engineering at the University of Texas at El Paso. During his Ph.D. studies, he has published two peer-review journal articles as the first author. Also, he has published seven peer-review journal articles, two peer-review book chapter and one journal review article, all of them as a co-author. He is also a member of the University of California’s Center for Environmental Implications of Nanotechnology (UC CEIN) and the American Chemical Society (ACS). He has presented his research at six different local or national conference meetings.

While pursuing his Ph.D., Mr. Chaoyi Deng worked as a Teaching Assistant in the Department of Environmental Science and Engineering. Also, he was the president (2015-2016) of UTEP Chinese Students and Scholars Association (CSSA). He also served as a volunteer in several campus research events, such as research expo and COURI.

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