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Effects Of Unweathered Or Soil Weathered Copper-Based Nanoparticles And Compounds On Soil Grown Bell Pepper (capsicum Annuum) And Spinach (spinacia Oleracea) Plants

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EFFECTS OF UNWEATHERED OR SOIL WEATHERED COPPER-BASED NANOPARTICLES AND COMPOUNDS ON SOIL GROWN BELL PEPPER (*CAPSICUM ANNUUM*) AND SPINACH (*SPINACIA OLERACEA*) PLANTS

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Dedication

I dedicate this work
to MA and PAPA
for their unconditional love and support
to my brother ASHISH
to my sister-in-law ASHIMA
and to my loving niece ANAHITA
for being an integral part of my life

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Abstract

Engineered nanoparticles (ENPs) find large scale industrial application because of their promising chemical, physical, electrical, magnetic, optical, and electronic properties. Copper-based ENP are used as gas sensors, in catalysts, electronics, semi-conductors, and in agriculture. They have been useful as agricultural amendments in the form of pesticides, herbicides, and fertilizers because of their antifungal and antibacterial properties. They also find application in electronics and sensors used in precision agriculture. Due to their massive production and use, ENPs are likely to be ubiquitous in the environment around us in the near future. Application of biosolids for fertilization and water from wastewater treatment plants for irrigation are other possible routes of ENP exposure to plants. Several studies have shown that copper-based ENPs disturb plant physiology and development; however, practically no work and very little work has been done on bell pepper (*Capsicum annuum*) and spinach (*Spinacia oleracea*) plants. Bell pepper is a worldwide consumed vegetable very rich in carotenoids, especially zeaxanthin, a powerful antioxidant. Spinach is another important vegetable consumed widely across cultures as a source of iron and pro-vitamin A. This research project is aimed to understand the effects of copper based compounds on bell pepper plants at half and full life cycle. It also analyzes the effects of soil weathering of the copper based nanoproducts on spinach plants.

The investigation included three parts. The very first part consisted of culturing bell pepper plants until reaching physiological maturity (90 days), in soil amended with nano CuO (nCuO), bulk CuO (bCuO), and ionic copper ($CuCl_2$) at 0, 125, 250, and 500 mg/kg. Ionic copper significantly decreased the gas exchange parameters, evapotranspiration, stomatal conductance, and photosynthesis by an average of 41 %, 59%, and 38%, respectively, compared to the other treatments at select concentrations ($p \leq 0.05$). Except for bCuO at 500 mg/kg, at 250 mg/kg and

above, the three compounds significantly increased root Cu (196%, 184%, and 184%), respect to control. Only 500 mg/kg ionic Cu gave significantly higher root Cu compared to the other Cu treatments. Additionally, at 125 mg/kg, leaf P was 41% lower for nCuO, against the bCuO treatment. At 500 mg/kg, nCuO reduced Zn by 55% in leaf and 47% in fruit, compared to control ($p \leq 0.05$).

The second part encompassed the evaluation of nCu and bCu species on agronomical and biochemical traits of bell pepper plants. This part was conducted in 2 phases, namely, vegetative stage [(VS)/half life cycle study (45 days)] and reproductive stage [(RS)/full life cycle study (90 days)]. Thirty day-old seedlings were transplanted into soil amended with 0 (control), 62.5, 125, and 500 mg Cu/kg and evaluated at the two phenological stages. At the VS, plants exposed to 500 mg nCu/kg had longer (58%) and heavier (187%) roots, compared to control ($p \leq 0.1$). At such growth stage, all plant tissues showed significantly higher Cu concentration from bCu treatments. Contrarily, at RS, Cu concentration in leaves of plants exposed to 500 mg nCu/kg was higher by 1510%, compared to bCu ($p \leq 0.05$). Additionally, 500 mg/kg nCu increased photosynthesis (42%) and stomatal conductance (51%), while 62.5 mg/kg increased evapotranspiration (31%), compared to bCu counterpart ($p \leq 0.05$).

In the third part, the ENPs were weathered/aged for 5 weeks in soil before spinach seedling transplantation. The resultant effects on the agronomical, physiological, and biochemical parameters, especially on the nutrient quality were evaluated. Plants were exposed for five weeks to freshly prepared or soil-aged (35 days) nCuO, bCuO, or $CuSO_4$ suspensions/solutions at 0 (control), 400, 400, or 40 mg/kg, respectively. Foliar health, gas exchange, carotenoid, chlorophyll, catalase and ascorbate peroxidase enzymes, as well as element bioaccumulation were evaluated. Foliar biomass was higher in UW control (84%) and in UW ionic treatment (87%), compared to

the corresponding W treatments ($p \leq 0.1$). Shoot Cu bioaccumulation for both W and UW (nano and bulk) CuO were significantly higher than control but showed no effect of weathering. No significant differences were observed in the root Cu bioaccumulation. At 40 days post transplantation, the intercellular CO_2 concentration was lower by 28% for W nCuO treatment, compared to W control ($p \leq 0.1$). Root catalase activity was higher by 110% in UW bCuO treatment, compared to the W counterpart; it was also higher for W ionic treatment by 216%, compared to the UW one ($p \leq 0.05$). At 20 days post transplantation, W nCuO gave 56% and 57% lower carotenoid, compared to W control and to UW counterpart, respectively ($p \leq 0.05$). At 10 day exposure period, Chl a for UW control was higher by 72% than W control ($p \leq 0.05$); conversely, at 30 days, it was higher for W by 91% than the UW control ($p \leq 0.05$).

This work was especially focused on the nutritional quality of the fruit/leaf, since that is the portion of plant consumed by humans. The findings indicate that depending on the plant species, growth media, and specific dosage of copper-based ENPs, they could be beneficial or detrimental to plant health.

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Chapter 1: Introduction

1.1 Background

Time has progressively raised awareness in the scientific community about the widespread use of engineered nanoparticles (ENPs) in the manufacture of engineering materials, cosmetics, pesticides, electronics and other household goods. According to the British Standards Institution, a nanomaterial is defined as any material with one dimension less than 100 nm (Klaine et al., 2008), while ENP, the chemical constituents of engineered goods, have at least two dimensions less than 100 nanometers. ENPs are used on an industrial scale for their extraordinary magnetic, electrical, optical, and chemical properties. They offer increased reactivity, the high surface energy, high redox activity (Wang et al., 2013) and the electronic property of quantum confinement (Ma et al., 2010). Most of the ENPs end up in soil after end user application, through biofertilizers (biosolids), via irrigation water from waste water treatment plants, and by pest treatment. Studies have shown that ENPs may be retained in soil and accumulate in the edible roots, but they can also be translocated from roots to the aboveground edible parts (Wang et al., 2012), entering into the food chain. ENPs' minute size allows their easy diffusion through tissue or cell membranes, causing several alterations that make the plants even more susceptible to toxicity.

Copper-based ENPs are widely used in paints and pigments, electronics, medical uses, cosmetics, and as catalysts. They are an essential part of present day agriculture and find use as active ingredients in fungicides, herbicides, pesticides, and fertilizers (Apodaca et al., 2018, Tamez et al., 2019). Copper-based ENPs are used in nano-sensors and nano- barcodes to serve as an aid in precision farming. There is a limited understanding of the environmental fate, transport, and toxicity of Cu NPs. Hence, this study was undertaken.

Copper is a vital micro nutrient in plants. It is part of a number of proteins, especially the one involved in photosynthesis called plastocyanin, and the one involved in respiratory electron transport chains called cytochrome oxidase. In excess, it negatively affects the membrane permeability, enzyme activities, protein synthesis, production of reactive oxygen species (ROS), chlorosis, plant growth, photosynthetic, and respiratory processes; it could even initiate senescence (Vinit-Dunand et al., 2002, Nair et al., 2014). Copper is also an important player in the enzyme level redox reaction. The limited work on phyto-toxicological influence of copper ENPs does not allow for regulations to keep a check on their production and use. Data from this and similar studies would go toward putting the needed regulations in place. Understanding the fate and transport of copper-based ENPs in soil is of considerable value to human and ecological health (Sagee et al., 2012).

Bell pepper (*Capsicum annuum*) is one of the most popular vegetables around the globe and is said to have originated in Mexico and Central America. It belongs to the nightshade family of vegetables, potato and eggplant being other members. The world green pepper production (along with *Capsicum frutescen* and *Pimenta officinalis*) was 34.5 million tons in 2016 and has risen at a 3% annual rate for the last decade (http://www.fao.org/faostat/en/#data/QC; Date accessed: 08.24.2018). A phenolic compound capsaicin gives it the pungent and chili flavor which chemically is 8-Methyl-N-vanillyl-6-noneamide (Abu-Zahra, 2012). Bell peppers are found to be rich in carotenoids, vitamin C, and antioxidants. The carotenoid and the vitamin C content in the fruit improves as it ripens with time.

Spinach (*Spinacia oleracea*) is another important green leafy vegetable consumed widely across cultures. It comes from the Chenopodiaceae family and originated in Central and Western Asia. A total of 26 million tons of spinach was produced the world over in 2016 and its production

has been rising at an average annual rate of 6% since 2006 (http://www.fao.org/faostat/en/#data/QC, last accessed: October 12, 2018). It is eaten raw as salad as well as cooked. It is a vital source of vitamin A, vitamin C, and iron in the dietary intake.

1.2 Chemistry of copper nanoparticles

Copper oxide nanoparticles (CuO NPs) are the simplest of copper compounds and possess a mono-clinical crystal lattice and rectangular morphology. They are used widely in semiconductors because of a narrow bandgap. Their highly ionic nature makes them a popular antimicrobial. They are considered harmful to aquatic life for the same reason. Copper NPs, at the same time, possess a round morphology and cubic crystal lattice. Cu has a high redox potential and can be present in multiple oxidation states. Copper NPs are brownish black in color and are considered highly inflammable (Gawande et al., 2016, https://www.azonano.com/article.aspx?ArticleID=3271, last accessed: November 7, 2018). Copper based ENPs are considered a good substitute of Ag NPs as antimicrobials, Cu being cheaper comparatively (Ren et al., 2009). Karlsson et al. (2008) tested the toxicity of a number metal oxide NPs against that of carbon nanotubes (CNT) on human lung epithelial cell line and found CuO NPs to be most toxic. They caused significant oxidative stress and DNA damage in the cell line. This testifies the very ionic nature of CuO.

1.3 Literature review: interaction of copper nanoparticles with plants

Studies have shown the phytotoxic potential of copper-based ENPs. A toxicity study was conducted to assess the effect of copper exposure in leaves of cucumber plants (*Cucumis sativus* L.). The effect of about 10 µg/g of copper particles fed to the plant through nutrient solution caused a reduction in photosynthesis in mature leaves and exhibited a marked reduction in leaf area in the younger leaves (Vinit-Dunand et al., 2002). Another study showed that both micro and nano

copper species (0-500 mg/L) were found to be highly toxic, and reduced the growth and transpiration by 60-70% in squash (*Cucurbita pepo*) (Musante & White, 2012). Mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*) were tested with copper nanoparticles using plant agar test at concentration 1-1000 mg/L. Inhibition of growth was observed in the two plants, but mung bean was much more sensitive and responsive to the effects of Cu ENPs (Lee et al., 2008). Another study with wheat showed that, at 500 mg/kg, CuO ENPs negatively affected the shoot growth, along with lipid peroxidation, oxidized glutathione in roots and reduced chlorophyll content in shoots (Dimkpa et al., 2012). These researchers concluded that the effects of copper-based ENPs are plant species-dependent. In soybean (*Glycine max* L.), exposure to 500 mg/L CuO ENPs hampered the chlorophyll content, shoot growth, and weight of the plants. It also increased hydrogen peroxide levels, peroxidase activity, and the lignin in root cells (Nair & Chung, 2014). In a seedling stage study on mung bean, cultured in Murashige and Skoog medium under CuO ENPs exposure (0-500 mg/L), total chlorophyll, root and shoot length, and biomass significantly reduced (Nair et al., 2014). In hydroponically grown lettuce (*Lactuca sativa*), core-shell Cu/CuO at 10 and 20 mg/L, reduced water content, root length, and dry biomass. There was also an increase in Cu, Al, and S but decrease in Mn, P, Ca, and Mg under the treatments in the plants (Trujillo-Reyes et al., 2014). Hong et al. (2014) studied the effect of several Cu species in hydroponically grown lettuce and alfalfa at 0-20 mg/L. They observed that the Cu-based ENPs reduced the plant root length by 49% in both plant species. They helped raise the Cu, P, and S content in alfalfa shoots and reduced the P and Fe content in lettuce shoots at various treatments. The enzyme assays indicate reduced catalase activity in alfalfa roots and shoots and increased ascorbate peroxidase activity in roots in both plant species (Hong et al., 2014).

Zuverza-Mena et al. (2015) observed that 20 mg nCu/kg treatments significantly reduced the seed germination in cilantro. Soil borne 80 mg nCu/kg and 20 mg/kg bCuO significantly hampered the cilantro shoot length, compared to control. In another study, cucumber plants were grown hydroponically in nCu (10 and 20 mg/L) for a metabolomics analysis by Zhao et al. (2016). These researchers noticed metabolic activity in the root exudates, indicating metal related stress that interfered with root nutrient uptake. The root activity showed an influx of phenolic compounds, amino acids, ascorbic acid, and a drop of citric acid, as a defense mechanism against metal toxicity (Zhao et al., 2016). In a following study, Zhao et al. (2016b) noted an altered metabolites profile in cucumber fruit under 400 and 800 mg nCu/kg of soil treatments. At the said treatments, nCu up-regulated proline, xylose, glycine, valine, and benzoic acid, while down-regulated citric acid, myo-inositol, ornithine, and 1-kestose. Ogunkunle et al. (2018) exposed cowpeas (*Vigna unguiculata*) plants to 0-1000 mg nCu/kg via soil for 65 days and observed 250% higher Cu bioaccumulation in the seeds, compared to control, along with altered enzyme activity. Du et al. (2018) examined the effect of metallic Cu at 0-200 mg Cu/kg in soil grown oregano plants and found that micron sized Cu reduced the root biomass, root length, and water content at varying treatments.

Few studies have come up in the recent past demonstrating the effect of soil weathered ENPs on crop plants. Servin et al. (2017) conducted an experiment on the effects of weathered vs unweathered CuO compounds on lettuce plants. Plant growth in seventy day weathered Cu compounds led to a 214% higher Cu bioaccumulation in the root in the aged vs unaged nCuO treatments. Both ionic and bCuO weathered treatments had an opposite effect to weathered nCuO on Cu bioaccumulation. Further trophic transfer tendencies and the resultant effects of weathered nCuO were examined. Another study analyzed the effects of weathered vs unweathered ZnO NPs

and ionic Zn on wheat plant examining Zn bioaccumulation in the plant tissue, especially the grain and productivity of the cereal (Dimpka et al., 2018). The unaged compounds gave a better bioaccumulation both in the plant tissue and in the grains as compared to the aged ones.

1.4 Experimental workflow

The experimental procedure encompassed sowing the seeds in starting mix at the green house, preparing potted soil with the different Cu treatments, seedling transplantation, and plant culture for the designated time under the green house conditions, harvesting, and analyses at the laboratory. This study was performed in three different parts/experiments.

1.4.1 Part I

The first part included cultivation of bell pepper plants in farm soil characterized as silty loam. General purpose plastic pots containing soil amended with measured amount of copper/kg of soil (Tables 1.1, 1.2, 1.3) were used for the purpose of growing the plants. One month-old seedlings were transplanted to pots containing the copper amended soil. Post transplantation, the growing plants were watered regularly with 100 ml water per kg of soil depending on the growth stage of the plant. The plants were irrigated with fertilizer solution, i.e., 15-5-15 ratio of N-P_2O_5-K_2O, pH: 5.8, EC: 1.00 mS/cm. They were monitored closely to check for any pests or plant disease. They were sprayed with Avid 0.15 EC, chemically composed of Abamectin to treat for aphids or white flies. The plants were closely monitored through the growing phases until they reached the flowering stage around forty five (45) days post transplantation. Ninety days post transplantation, at the mature and fruiting stage, gas exchange was measured in the plants and harvesting was conducted. Different agronomical parameters like stem length and weight, leaf count and weight, total foliar area, fruits count and weight, root length and weight were noted. Different plant tissues were segregated, washed in 0.01 M HNO_3 and water, air-dried, and oven

dried for further elemental analysis via ICP-OES to check the uptake and translocation of Cu in the plant, along with the effect on other minerals.

Table 1.1: Reproductive stage study (CuO) with bell pepper plants.

Treatment	**Treatment**	**Treatment**	**Replicates**
Control	Control	Control	4 replicates each
62.5 mg/kg nCuO	62.5 mg/kg bCuO	62.5 mg/kg $CuCl_2$	4 replicates each
125 mg/kg nCuO	125 mg/kg bCuO	125 mg/kg $CuCl_2$	4 replicates each
250 mg/kg nCuO	250 mg/kg bCuO	250 mg/kg $CuCl_2$	4 replicates each
500 mg/kg nCuO	500 mg/kg bCuO	500 mg/kg $CuCl_2$	4 replicates each

1.4.2 Part II

The second part dealing with the effects of size of metallic Cu, nano and bulk, on bell pepper plant at half life cycle vs full life cycle was conducted the exact same way and under the exact same conditions as part I. As given on Table 1.2, the first set of the treatments in the experimental setup were harvested at 45 days post transplantation, when the plants had just started flowering. These were grown in 2 kg soil for half life cycle measurements. The second set of the treatments, as given on Table 1.3, were harvested at the mature fruiting stage of the plants at 90 days post transplantation. It is important to note here that all the plant treatments on Tables 1.2 and 1.3 were started simultaneously. The reproductive stage study (90 day exposure) in this part encompassed catalase and ascorbate peroxidase enzyme activity determination in leaves and root. It also involved fruit nutrient quality analysis that included carotenoid, chlorophyll, vitamin C, and total sugar content. All the other procedures followed were same as those mentioned under part I.

Table 1.2: Vegetative stage study (Cu) with bell pepper

Treatment	Replicates
Control	3 replicates
62.5 mg/kg nCu	3 replicates
500 mg/kg nCu	3 replicates
62.5 mg/kg bCu	3 replicates
500 mg/kg bCu	3 replicates

Table 1.3: Reproductive stage study (Cu) with bell pepper

Treatment	Treatment	Replicates
Control	Control	3 replicates
62.5 mg/kg nCu	62.5 mg/kg bCu	3 replicates each
125 mg/kg nCu	125 mg/kg bCu	3 replicates each
500 mg/kg nCu	500 mg/kg bCu	3 replicates each

1.4.3 Part III

The experimental setup for the third part/experiment is as given on Table 1.4. This part included weathering of Cu based chemicals at field capacity for five weeks. The five week old seedlings were then transplanted in weathered as well as fresh Cu treatments simultaneously. The Cu compounds were anticipated to undergo surface chemistry and other physico-chemical transformations in the soil during the aging period. Their effects might thus vary from the freshly applied treatment counterparts. Additionally, this experiment included gas exchange monitoring, leaf catalase and ascorbate peroxidase, SPAD, chlorophyll, and carotenoid content measurements

every 10 days over the 30 day life cycle of the spinach plant. Plant harvest procedure was the same as given under part I.

Table 1.4: Soil weathered Cu compounds with spinach plant

Treatments	**Soil with weathered NPs**	**Soil with fresh NPs**
Control	3 Replicates	3 Replicates
400 mg/kg nCuO	3 Replicates	3 Replicates
400 mg/kg bCuO	3 Replicates	3 Replicates
40 mg/kg Ionic copper, $CuSO_4$	3 Replicates	3 Replicates

1.5 Hypotheses and problem statements

These studies were performed under the following working hypotheses that:

1) The nano sized copper species induce more toxicity in plants as compared to the micro sized copper species. Toxicity implies a reduction or an influence on the plant nutrient content.

2) Weathering of Cu NPs induce increased toxicity in plants.

We seek to answer the following research questions:

1) To what extent do the Cu NPs induce phyto-toxicity in bell pepper and spinach plants, whether they have an influence on the nutrient content and plant health?
2) Comparative study of the toxic effects of nano copper compounds *vs.* the micro or bulk copper compounds *vs.* the ionic compound.
3) What is the effect of weathering/aging of copper NPs in agricultural soil and the consequential effect on plants?

The effect of weathering on the phytotoxicty of Cu ENPs is a relatively new topic. This research will serve as a precedent for future work and will give us an insight into the widening horizons of plant nano-bio-interactions research.

1.6 Specific objectives

Specific objectives of this research included:

1) To assess the physiological and biochemical effects of particle size and concentration on the toxicity elicited by the copper-based chemicals on edible plants (bell pepper and spinach).

2) To assess the physiological and biochemical effects of soil weathered copper-based ENPs on spinach plant health and nutrition.

Chapter 2: Impacts of copper oxide nanoparticles on bell pepper (*Capsicum annuum*) plants: A full life cycle study[1]

2.1 Introduction

Engineered nanomaterials (ENMs) are one important class of chemicals being studied for their effects on ecology and human health. ENMs have an ever rising application in industry for their unique technical properties (Peralta-Videa et al., 2011). Among these, Cu-based nanoparticles (NPs) are used as additives in lubricants and printer's ink, in the manufacture of semiconductors and lithium ion batteries, as heat insulators, and as catalysts (Chen et al., 2006, Wang et al., 2012, Bondarenko et al., 2013, Zuverza-Mena et al., 2015). Recent estimates indicate that the world production of Cu-based NPs has reached 200 tons/year (Keller et al., 2013). Leaching and abrasion from various consumer products could make the Cu-based NPs ubiquitous contaminants and present unknown risks of exposure and toxicity. Cu-based NPs also have agricultural applications due to their antimicrobial properties (Bondarenko et al., 2013, Cheng et al., 2016). In addition, the application as bio-fertilizers of wastewater, sewage sludge, and bio-solids to the agricultural fields presents a possible route of exposure to the plants (Zuverza-Mena et al., 2017).

There are limited number of studies with Cu-based NPs in plants. CuO NPs (100 - 2000 mg/L) have shown toxicity to seed germination and root elongation in lettuce, radish, and cucumber (Wu et al., 2012). Another study illustrated reduced root length, moisture content, and dry biomass in lettuce plants exposed to 10 and 20 mg/L nano Cu/nano CuO (nCu/nCuO) in hydroponics (Trujillo-Reyes et al., 2014). Zuverza-Mena et al. (2015) reported that nCuO, bulk/micro CuO (bCuO) and copper chloride ($CuCl_2$) reduced cilantro (*Coriandrum sativum*)

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[1]Reprinted from Rawat, S., Pullagurala, V. L., Hernandez-Molina, M., Sun, Y., Niu, G., Hernandez-Viezcas, J. A., et al. (2018). Impacts of copper oxide nanoparticles on bell pepper (capsicum annum L.) plants: A full life cycle study. *Environmental Science: Nano, 5*(1), 83-95.

germination at both 20 and 80 mg of Cu/kg of soil but only bCuO reduced shoot elongation (at both concentrations) and chlorophyll content (at 20 mg/kg). Shi et al. (2011) (at 1 mg/L) and Lalau et al. (2015) (at 0.1, 1, and 10 g/L) found similar results regarding inhibition of chlorophyll and plant growth on duckweeds (*Landoltia punctata*) with nCuO. Studies conducted at the initial growth stage of different plant species grown under nCuO exposure (variable concentrations) indicated possible degradation of DNA and excessive production of reactive oxygen species (ROS) (Atha et al., 2012, Dimkpa et al., 2012, Lee et al., 2013, Nair et al., 2014, Nair et al., 2014). Hong et al. (2015) reported that nCuO reduced the root length at 5, 10, and 20 mg/L dosage and adversely affected the nutrient content and enzyme activity in alfalfa and cucumber plants.

Bell pepper (*Capsicum annuum* L.), is a warm weather crop widely consumed all over the world. A phenolic compound, capsaicin (8-Methyl-N-vanillyl-6-noneamide), gives it the pungent and chili flavor (Abu-Zahra, 2012). Bell peppers are rich in carotenoids, vitamin C, and antioxidants. Through 2007-2011, California was the biggest producer (51%) of bell peppers in the USA, followed by Florida (26%) (https://www.ers.usda.gov/webdocs/publications/39535/41021_vgs353sa1.pdf?v=42125, accessed September 2017). The world production and hence the consumption of fresh bell peppers (along with *Capsicum frutescen* and *Pimenta officinalis*) has consistently risen at a rate of about 3% every year over the last decade, with world production of about 32 million tons in 2014 (http://www.factfish.com/statistic-country/world/chillies%20and%20peppers%2C%20green%2C%20production%20quantity, accessed June 2017).

A limited number of studies have evaluated full life cycle NP exposure to plants. To the authors' best knowledge, no phytotoxicological studies have been conducted with chili or bell

pepper plants, especially in natural soil. This life cycle study was aimed to differentiate the physiological and agronomical effects of different particle size and species of Cu based chemicals on bell pepper plants. Treatment effects on ionome homeostasis, physiological, and agronomical parameters were evaluated using biochemical and spectroscopic techniques. Through this research we intend to contribute to the existing body of work on food safety about the possible influences of engineered nanoparticles on the nutritional quality of produce.

2.2 Method and material

2.2.1 Characterization of the copper-based chemicals

Nano-particulate copper oxide, bulk copper oxide, and reagent grade copper chloride salt were obtained from Sigma Aldrich (St. Louis, MO). Physico-chemical properties, zeta potential, hydrodynamic diameter, copper content by weight, morphology and additional properties are shown in Table 2.1.

2.2.2 Soil and treatments

The plants were grown in agricultural soil collected at a field in Socorro, TX (latitude: N 31°41' 30.705” and longitude: W 106°17' 13.936”, elevation: 1,115 m asl). A Malvern Mastersizer Hybrid 2000G (Malvern, Worcestershire, UK) was used to characterize the soil, which was silty loam (65% silt, 20% sand, and 15% clay) (Ryżak & Bieganowski, 2011).The soil pH in DI water and in $CaCl_2$ solution slurry were 7.4 and 7.5, respectively. The pots for transplantation were prepared by amending the soil with nCuO, bCuO, and $CuCl_2$ at 0 mg/kg (used as control), 62.5, 125, 250 and 500 mg of Cu/kg of soil, four replicates each and one plant per pot. Each pot contained 5 kg natural soil homogenized with an aqueous suspension/solution of the copper-based chemicals, prepared by stirring in DI water for 30 min. The copper chemicals were suspended/diluted in enough water to have the soil at about 60% of the field capacity. While

preparing the substrate, the contaminant homogeneity was ensured by a thorough manual mixing of the soil with the chemical suspension for 20 min.

The concentration range 62.5 mg/kg – 500 mg/kg was selected for its environmental relevance. As per USGS (https://pubs.usgs.gov/pp/1270/pdf/PP1270_508.pdf, accessed September 2017), the average copper concentration in various soils is about 30 mg/kg dry weight. The Cu concentration in pesticides is up to 50 mg/kg, in fertilizers about 300 mg/kg, and in sludge up to 3000 ppm dry weight. Fly ash has Cu concentrations ranging from 300 mg/kg to about 2000 mg/kg (https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=206&tid=37, accessed September 2017). The concentration range most pertinent to agricultural fields was picked up. Additionally, similar concentrations have been used in previous investigations.

2.2.3 Copper release in soil solution

Copper concentration in soil solution was determined for nCuO and bCuO species. Copper chloride was not considered for the analysis because it is readily soluble in water. Three replicates each with 20 g of the experimental soil mixed with the chemical and 30 mL water to make 500 mg/kg aqueous suspension were shaken for 12, 24, 36, and 48 h. The samples were stationed upright for 4 h after rocking. Thereafter, 12 mL of the supernatant was separated and centrifuged at 5000 rpm for 15 min (Sorvall, Legend XR1 centrifuge, Thermo Fisher Scientific, Waltham, MA, USA). Ten milliliters (10 mL) of the subsequent supernatant was decanted and centrifuged again at 5000 rpm for 30 min. Another round of centrifugation was done with 8 mL of the resultant supernatant at 15000 rpm for 30 minutes. The process was repeated thrice in order to filter out the particles in suspension and have just the dissolved chemical (ions) in solution. The final 5 mL of the soil solution was analyzed by ICP-OES for the concentration of dissolved copper in each sample (Dimkpa et al., 2012, Bandyopadhyay et al., 2015, Mukherjee et al., 2015).

Standard reference material 1570a from the National Institute of Standards and Technology was used to validate the digestion method, obtaining a recovery of 100% ± 5.0%. For quality control of the ICP readings, every 25 samples, blank and a spiked sample containing Cu 10 mg/L was read. The average readings for Cu in the spiked sample was 9.8 ± 0.40 mg/L. The ICP-OES parameters used were as follows: nebulizer flow, 0.80 L/min; power, 1,400 W; peristaltic pump rate, 1.5 mL/min; flush time, 20 s; delay time, 20 s; read time, 10 s; and wash time, 60 s.

2.2.4 Plant culture at the greenhouse

The bell pepper seeds (California wonder 300 TMR) were planted in Sunshine Ready Earth REPS soil mix (55%-65% Canadian sphagnum peat moss, vermiculite, dolomite, lime). Thirty (30) days after growth in a controlled greenhouse environment, seedlings were transferred to pots with copper amended soil. Through the growth period, the plants were regularly watered with nutrient solution containing NPK in the ratio 15-5-15 at 1g/L; the pH of the solution was maintained at 5.8 and the electrical conductivity (EC) at 1.0 mS/cm. The plants were treated with pesticide Avid 0.15 EC, active ingredient abamectin (2% v/v) to protect the plant against aphid and white fly infestation. Throughout the experiment, the average day temperature at the green house was recorded as 27.2 ± 1.6 ˚C and the average night temperature as 25 ± 2.1 ˚C. The average daylight was recorded as 10.1 ± 3.1 $mol \cdot m^{-2} \cdot d^{-1}$. The relative humidity at the greenhouse was recorded to be 47.1 ± 10.6% for the experiment.

2.2.5 Gas exchange and chlorophyll measurement

The gas exchange data was collected by using a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury, Massachusetts, USA) just prior to harvest at 90 days post transplantation. Photosynthetic rate, stomatal conductance, and evapotranspiration were measured in the newest fully expanded leaf of each plant. The plants were well-hydrated before measurements taken between 1000 to 1400 h on sunny days, ensuring no cloud cover that could influence the readings

(Zhao et al., 2013). In addition, relative chlorophyll content was determined using a hand held single photon avalanche diode chlorophyll meter (SPAD, Minolta Camera, Japan) by taking the average value across ten leaves per plant.

2.2.6 Harvest and elemental analysis

Ninety (90) days post transplantation, the plants were mature at the fruiting stage. The total foliar area, total number of fruit, and their total weight were recorded for each plant. The total foliar area was measured by LI-3100C area meter (LI-COR Biosciences, Lincoln, Nebraska, USA). The separated samples were washed in 0.01M HNO_3 followed by DI water. After air drying, the fresh samples were oven dried at 60-70˚C for 72 h.

The dried plant samples were weighed to determine the dry biomass. Samples were ground and the powders were digested in a digiPrep hot block (SCP Science, Quebec, Canada) for further analysis. The Environmental Protection Agency (EPA) method 3051 was employed for digestion using plasma pure nitric acid (65%) and hydrogen peroxide (30%) (1:4). Inductively coupled plasma-optical emission spectroscopy (ICP-OES, Perkin-Elmer Optima 4300 DV, Shelton, CT) was used to determine the macro and micro elements (Ca, K, Mg, P, S, Fe, Zn, Cu, Mn, Ni, Mo) concentrations in the digested samples (Zuverza-Mena et al., 2015). Three blank sample carriers, two carriers with spinach as the standard reference material (NIST 1570a, Gaithersburg, MD) and three with samples spiked with 20 mg/L of the Cu NPs were all digested and included in the analytical process as part of the QA/QC (Hong et al., 2015).

2.2.7 Statistical analysis

All the data collected were analyzed using SPSS (IMB) program. The mean values from the controls and the treatments were compared among each other using one-way ANOVA followed by Tukey-Kramer multiple comparison test (SPSS 19.0 package, Chicago, IL). Results are

presented as mean ± standard error and were observed for statistically significant differences at $p \leq 0.05$.

2.3 Results and discussion

2.3.1 Soil dissolution study

The dissolution characteristics of nano and bulk species of CuO in soil solution is shown in Figure 2.1. The pristine Cu content present in the agricultural soil (obtained from control) has been deducted off of the measured values from the treatments to give us the absolute Cu ion concentrations (Figure 2.1). As seen in the figure, nCuO particles favored Cu dissolution in the soil environment over the bulk species. The ionic Cu content in soil solution from nCuO was 388%, 230%, 152%, and 188%, greater than that from bCuO at 12, 24, 36, and 48 h, respectively ($p \leq 0.05$) (Figure 2.1). The concentration of ionic Cu consistently increased over time for the two chemicals, more rapidly for nCuO than for bCuO. The Cu concentration in soil solution from nCuO at 48 h was 20% greater than that at 36 h, which in turn, was 31% greater than that at 24 h ($p \leq 0.05$). The Cu concentration in soil solution from bCuO at 48 h was 81% higher than that at 24 h, and at 36 h, it was 219% greater than that at 12 h of dissolution ($p \leq 0.05$). Some studies have shown the dissolution of Cu from bCuO and nCuO in different media. For instance, Dimkpa et al. (2012) reported no differences in Cu released from both CuO compounds suspended in deionized water and added to sand for a period of 14 days. In another similar study, Gunawan et al. (2011) found that in Luria-Bertani medium, there was a significantly higher leaching of Cu from nCuO, compared to bCuO. Gunawan et al. (2011) suggested that the curvature of the nCuO favored the Cu dissolution. The fact that there are very few such studies in soil solution makes comparison difficult. However, McShane et al. (2014) reported that in two different soils, nCuO showed significantly more Cu activity than bCuO, which in some ways supports the results found in the current study.

Referring to Table 2.1, we see that there are orders of magnitude difference between the nCuO and bCuO primary particle size, nCuO being much smaller. A smaller particle size offers a much larger specific surface area for contact with water (Bawa et al., 2016). This could be a reason for observing the difference in the Cu dissolvability between the two Cu species (Figure 2.1). Similarly, the hydrodynamic diameter was relatively smaller for nCuO against bCuO, which may have influenced the Cu dissolution.

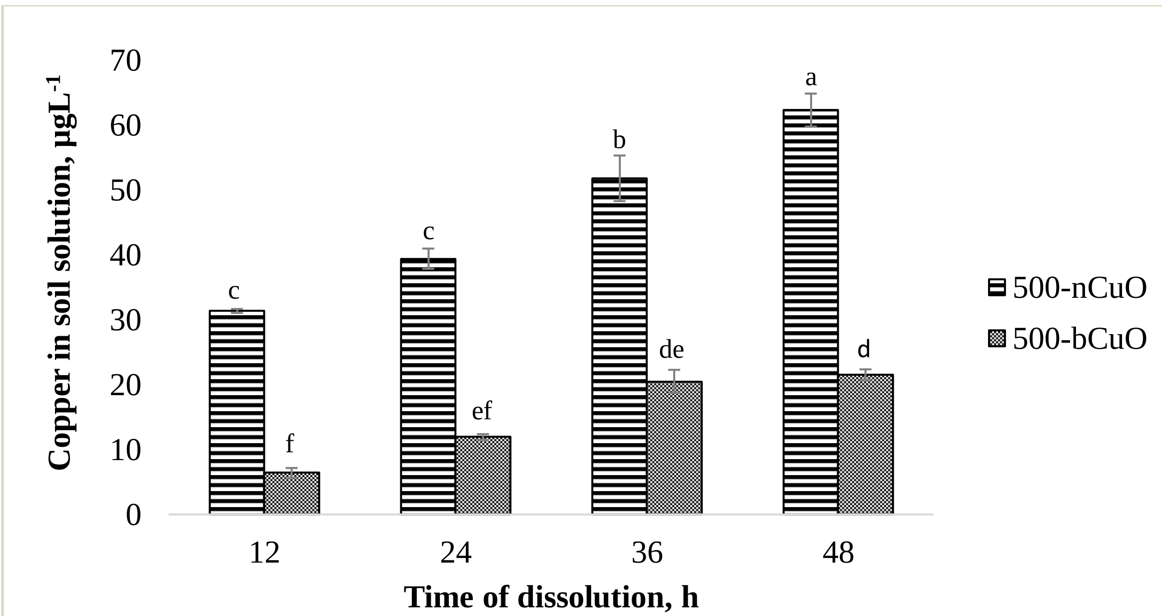


Figure 2.1: Relative dissolution of nCuO and bCuO in soil solution in DI water, deducting the amount of pristine copper present in natural soil. Data are averages of three replicates ± SE. Averages with a common alphabet are not significantly different with respect to each other, as per one-way ANOVA and Tukey test ($p \leq 0.05$).

2.3.2 Chlorophyll content

The chlorophyll measurements were made just prior to harvest. The differences from SPAD measurements were not statistically significant, indicating that the chemical treatments had no significant influence on the chlorophyll content in bell pepper plants.

2.3.3 Gas exchange

The gas exchange data including evapotranspiration (E), stomatal conductance (g_s), and photosynthesis (P_n) are shown in Figure 2.2. All the three markers of gas exchange displayed similar results and none of the treatments produced significant differences respect to control. However, a comparison between compounds showed that ionic Cu showed significant reduction in E, g_s, and P_n. compared to the other treatments. As shown in Figure 2.2 (a-c), at 62.5 mg/kg, ionic Cu reduced P_n by 38% and E by 44 % compared with nCuO, and by 39% compared with bCuO ($p \leq 0.05$). While at 125 and 500 mg/kg, it reduced E by about 40%, g_s by 58% and 59%, respectively, compared to bCuO ($p \leq 0.05$). Moreover, at 62.5 and 250 mg/kg, ionic Cu reduced g_s by 65% and 55%, compared with nCuO ($p \leq 0.05$).

The negative responses observed in ionic treatments, mainly at 500 mg/kg, could be due to the higher uptake and translocation of Cu to the leaves (Figure 2.2). The response at lower concentrations could be due to the rapid interaction of Cu or Cl^- with the plants, since both ions were available for plant uptake at a very early growth stage. Chloride has been shown to be toxic to several plants including tomatoes, beans, and barley, among others (Eaton, 1942). On the other hand, bulk-and-nano-CuO had a slower release of copper ions into soil (Figure 2.1), which likely led to slower Cu uptake and translocation (Keller et al., 2017). Julich and Gath (2014) conducted a sorption study to compare the behavior of CuO NPs *vs.* ionic Cu in different soil types. They observed that nCuO had a much stronger tendency to bind with different soil fractions, compared to ionic Cu species. In the current study, the concentration of Cu ions released from bCuO was

significantly different than that from nCuO, as per the soil dissolution study (Figure 2.1); however, they had similar effects on gas exchange parameters. The gas exchange measurement was taken just one time through the growth period of the plant, just prior to harvest. The results could be the cumulative effect of the exposure to the chemicals through the growth period, especially to the ionic Cu treatment.

Sekine et al. (2017) reported that Cu-based NPs dissolve in soil, and independent of the Cu form added to the soil, in the long term, the Cu species from dissolved Cu and Cu-based NPs was the same in different soils. This could have happened in our work, since the effects of the Cu form on most measured parameters were low.

Table 2.1: Characterization of nCuO and bCuO particles used in this study (Hong et al., 2015, Lin et al., 2015)

Property	**nCuO**	**bCuO**
Primary particle size (nm)	20 – 100	200– 2000
Hydrodynamic diameter (nm) [a]	280±15	376±26
Zeta potential (mV) [a]	-34.4±0.5	-42.7±0.153
Cu content (wt. %)	74.3	79.7
Purity (%)	88.3±1.3	92.8±1.1
Other elements present	O, C	O
Shape, Morphology	Rhombus, irregular	Prism, irregular
Main Copper phase	CuO	CuO
Crystal structure	Monoclinic	Monoclinic

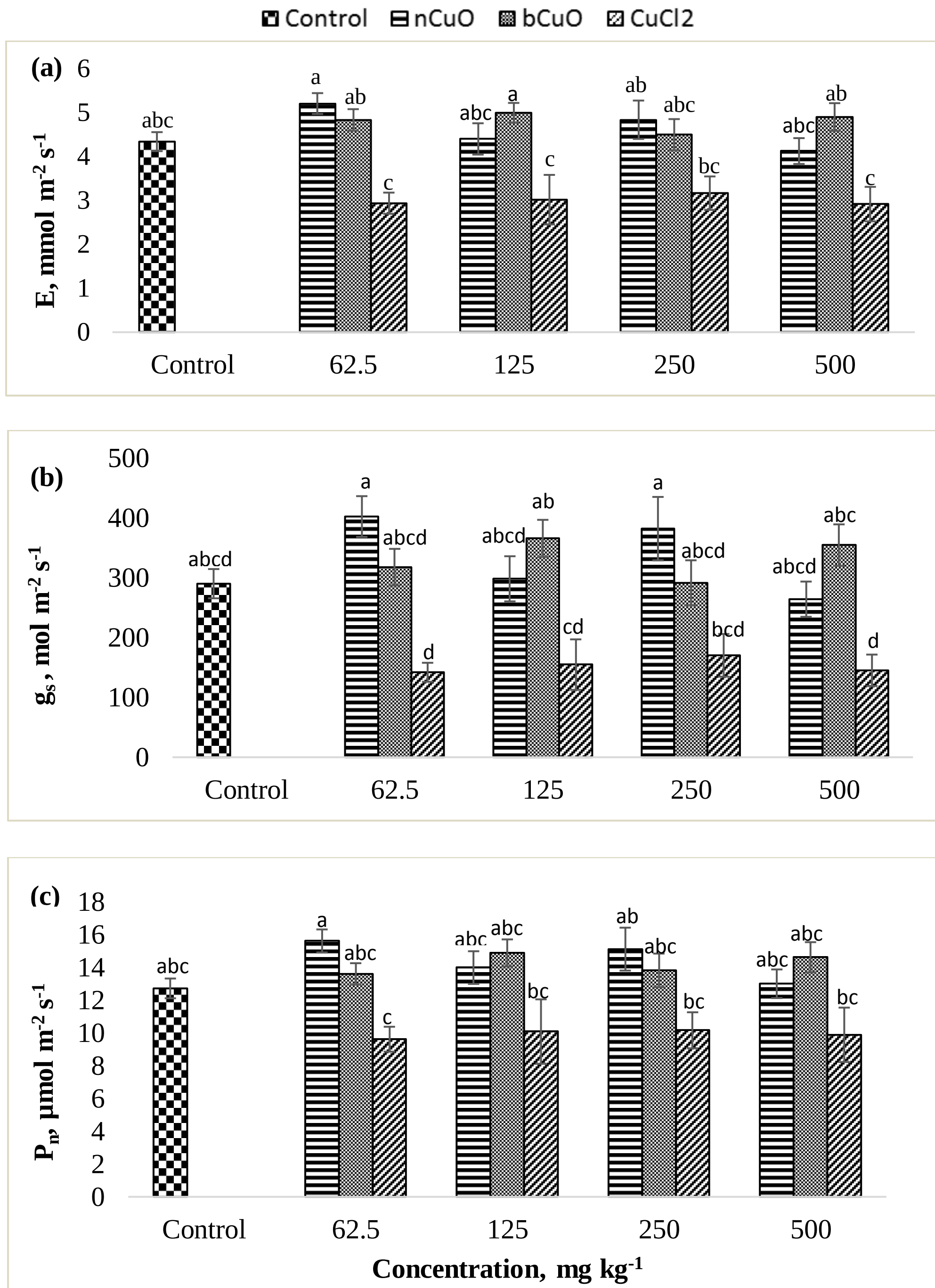


Figure 2.2: Comparison of the gas exchange parameters (a) evapotranspiration, (b) stomatal conductance, and (c) photosynthesis in bell pepper plants cultivated for 90 days in natural soil amended to nCuO, bCuO, and $CuCl_2$ at 0, 62.5, 125, 250, and 500 mg Cu/kg. Data are averages of four replicates ± SE. Averages with a common alphabet are not significantly different with respect to each other, as per one-way ANOVA and Tukey test ($p \leq 0.05$).

2.3.4 Copper uptake and translocation

Copper uptake

The experimental soil had 17.34 ± 0.53 mg of total Cu/kg soil, as given on Table A2.6 (Annex I). The concentration of Cu in bell pepper roots is shown in Figure 2.3c. All Cu treated plants had significantly more Cu in the root, compared with control. Similar results have been reported in previous studies with *Bromus carinatus* (Hook and Arn.), corn, cucumber, radish, rapeseed, ryegrass, wheat, mung bean, duckweeds, and lettuce (O'Dell et al., 2007, Lin & Xing, 2007, Lee et al., 2008, Shi et al., 2011, Trujillo-Reyes et al., 2014). In bell pepper, however, the differences were statistically significant only at higher concentrations such as 250 and 500 mg/kg, except for bCuO at 500 mg/kg. Increases at 250 mg/kg were about 196, 184, and 186%, respectively, for nCuO, bCuO, and ionic Cu, compared with control ($p \leq 0.05$). Interestingly, there were no differences between compounds at 250 and 500 mg/kg, except for ionic copper at 500 mg/kg. At this concentration, roots exposed to ionic copper had about 45% more Cu than that at 250 mg/kg. At 500 mg/kg of nCuO and of ionic Cu, root Cu was higher by 239% and 437%, respect to control ($p \leq 0.05$).

Several factors determine the accumulation of Cu in roots including the added concentration, soil properties, plant species, and root morphology (Monica & Cremonini, 2009). Metallothionein and phytochelatins are important organic complexes likely formed in plant root cells because of which, Cu, especially from nCuO, is retained in the root (Fernandes & Henriques, 1991). Our observations about the Cu in roots is contrary to what has been reported from sand grown wheat and soil grown cilantro (Dimkpa et al., 2012, Zuverza-Mena et al., 2015). The authors apparently did not see significant variation in Cu concentration in root samples under nCuO treatment. Very likely, variations in the response are due to differences pertaining to the plant species and the experimental design. Additionally, soft metal cation NPs are strongly affected by

inorganic and organic ligands (Lowry et al., 2012) that are abundant in agricultural soils such as the one used in this study.

Copper translocation to leaf and fruit

Concentrations of Cu in leaves and fruits are shown in Figures 2.3(b-a), respectively. No significant differences were observed between the responses from nCuO and bCuO treatments, at any concentration. At 125 and 250 mg/kg, bCuO showed higher leaf Cu than control (97% and 89%), while ionic copper increased leaf Cu at 250 and 500 mg/kg (129% and 116%), as compared to control ($p \leq 0.05$). In fruit, only bCuO at 250 mg/kg showed higher Cu concentration (51%) compared with control. The result observed with bCuO was probably due to its larger negative zeta potential, compared to nCuO (Table 2.1). The soil had predominantly a negative charge as did the CuO particles. The apparent repulsion between the two negative charges probably fueled the translocation of bCuO at certain concentrations (Lin et al., 2010). Moreover, nCuO probably underwent aggregation in the rhizosphere and the root tissues, thus resulting in a different behaviour than bCuO (Karlsson et al., 2006, Baalousha et al., 2008, He et al., 2008, Hotze et al., 2010). Under same pH conditions, aggregation is higher for smaller particles (Baalousha et al., 2008).

Tanslocation factors for Cu (TF, ratio of the elemental concentrations between leaf and root) are shown in Table A2.7, (Annex I). Among all the Cu treatments, 125 mg/kg bCuO gave the highest TF, followed by control and the lowest TF was given by 500 mg/kg $CuCl_2$. Bulk CuO allowed higher Cu translocation to fruit, apparently because of its relatively high negative charge (Table 2.1) and the soil negativity(Lin et al., 2010). A low TF for 500 mg/kg $CuCl_2$ needs further work for an explanation. Cu is an element with low mobility in soil (Nemecek et al., 2002), but forms very strongly bound chelates (Bañuelos et al., 1999, Sekine et al., 2017). The latter factor

tends to magnify for the nano species, since they tend to homoaggregate, along with heteroaggregation (Lowry et al., 2012). Further translocation of Cu takes place from the root to the fruit. Since none of the treatments affected fruit Cu (8-12 mg/kg) and the Cu dietary reference intake is 700-1000 µg/day (Dietary Reference, Food and Nutrition Board, Institute of Medicine), this suggests that none of the treatments compromised the Cu consumption through bell pepper exposed to nCuO, at the concentration tested, in a similar soil like the one used in this study.

The gas exchange responses from treatments (Figure 2.2) did not align with the leaf Cu accumulation (Figure 2.3b). On one hand, the leaf Cu concentration from both the bCuO and ionic Cu treatments significant increased, at varying concentrations, but they had different effects on gas exchange parameters. On the other hand, under similar Cu content from varying concentrations of ionic Cu and bCuO/nCuO, there were significant differences in gas exchange. This probably ocurred because gas exchange is influenced by other factors, besides the Cu concentration. Zhao et al. (2013) observed no significant changes in gas exchange in CeO_2 treated cucumber plants. They conjectured that the chemical adsorbed on the root surface exerted influence on the water transport through the plant system. It is possible that similar phenomena ocurred in bell pepper where nCuO aggregated and adsorbed in the soil rhizosphere and influenced the water uptake (Rico et al., 2011, Lowry et al., 2012, Sekine et al., 2017).

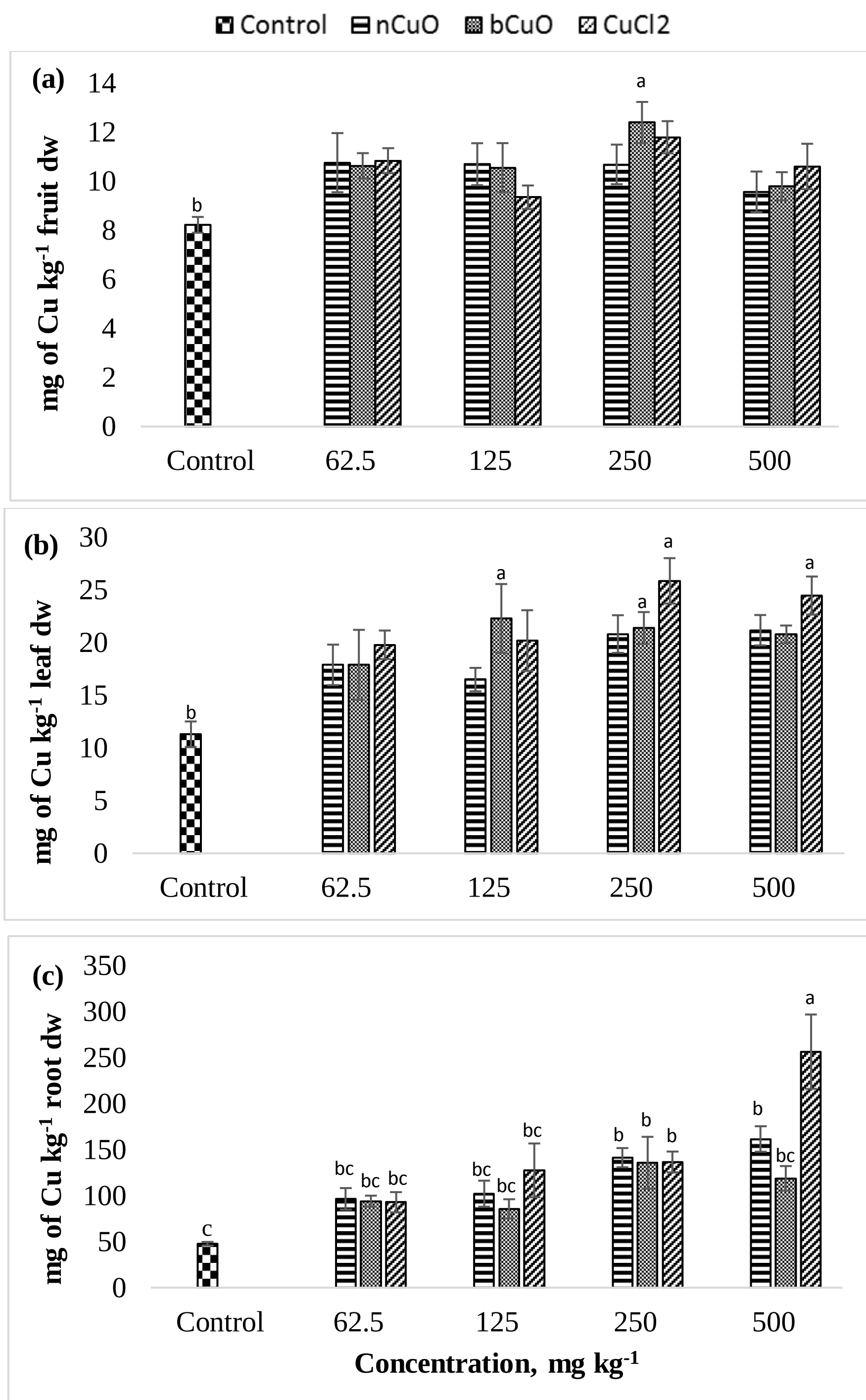


Figure 2.3: Copper concentrations in (a) fruit, (b) leaf and (c) root of bell pepper plants cultivated for 90 days in soil amended with nCuO, bCuO, and $CuCl_2$ at 0 (control)-500 mg of Cu/kg of soil. Data are averages of four replicates ± SE. Averages with a common alphabet are not significantly different with respect to each other, as per one-way ANOVA and Tukey test ($p \leq 0.05$)..

2.3.5 Absorption of essential elements

Out of all the elements analyzed in root, leaf, and fruit samples, only those with significant differences ($p \leq 0.05$), compared with control and among treatments, are discussed. The three Cu compounds were observed to have varying effects on the level of nutrients in plant tissues.

Root elemental content

Figure 2.4 shows the concentrations of elements that were altered in roots including phosphorus, sulfur, selenium, and molybdenum. Table A2.6 (Annex I) gives the background concentration of all the elements in native soil for reference.

Phosphorus: As shown in Figure 2.4a, only bCuO at 500 mg/kg altered P absorption in the root, which was reduced by 36%, respect to control ($p \leq 0.05$). As explained previously, the negativity of soil particles (Lin et al., 2010) could force the movement of the negatively surface charged bCuO to the roots, which can physically block the uptake of some elements. In addition, slowly released Cu ions from bCuO (Dimkpa et al., 2012) at the root-surface interface could be complexed with phosphate ions ($H_2PO_4^-$, HPO_4^{2-}), making P non-bioavailability for the plants (Zuverza-Mena et al., 2015). Hong et al. (2015) reported a reduction of root P in lettuce and alfalfa plants exposed to bCuO and nCuO. Ait Ali et al. (2002) had similar observations with roots of maize plant exposed to Cu. Safaya (1976), found an antagonistic effect between copper and phosphorus as nutrient elements in corn plants.

Sulfur: Sulfur concentration is shown in Figure 2.4b. As shown in this figure, only ionic copper at the highest concentration (500 mg/kg) altered S in root tissues, which increased by 71% with respect to control ($p \leq 0.05$). Sulfate transporters *Sultr 1; 1* and *Sultr 1;2* were likely expressed in the root at 500 mg/kg of ionic copper (Takahashi et al., 2000, Gigolashvili & Kopriya, 2014). Sulfur is the main component of organic protein complexes, like metallothionein and phytochelatins in the plant system. These complexes are the plant's response to metal toxicity and

play a vital role in cellular detoxification and metabolism (Cobbett, 2003). Cu from ionic copper most likely formed copper sulfate or organic complexes in the soil (Eaton, 1942), which were taken up by the roots and translocated further to the leaves and fruits (Flemming & Trevors, 1989).

Selenium: Concentration of Se in bell pepper root is shown in Figure 2.4c. As illustrated in Figure 4c, there was a great variability in the Se accumulation; however, only bCuO at 125 mg/kg showed statistically significant differences, compared to the other treatments. Root Se showed an inverted U shape profile, but in all cases, there were high error bars, which overlapped the response with the other treatments. Se in root samples at 125 mg/kg bCuO was higher by 3469%, compared with control, and by 1048%, compared with nCuO at the same concentration ($p \leq 0.05$). Selenium is a non-essential element for plants (Pilon-Smits, 2015). It is chemically very similar to sulfur, hence, shares metabolic pathways and transporters with it (Sors et al., 2005).

Molybdenum: Concentrations of Mo in roots of bell pepper are shown in Figure 2.4d. As shown in this figure, Mo was not detected in plants exposed to bCuO, at all concentrations (IDL: 37.8 µg/kg). Mo is taken by plants as MoO_4^{2-} (Nie et al., 2014), and bCuO had a high negative charge (-42.7 mV) that, very likely, repelled molybdate from the root surface. This could also have happened with nCuO at 500 mg/kg, which significantly reduced (about 88%) Mo uptake, compared with the ionic Cu treatment. Additionally, Mo is said to share some transporters with S and P (Nie et al., 2014). Mo uptake and transport at 125 mg/kg ionic copper treatment was probably supported by the P transporters because the P transport at the same treatment was not significant in the root (Heuwinkel et al., 1992, Gupta, 1997).

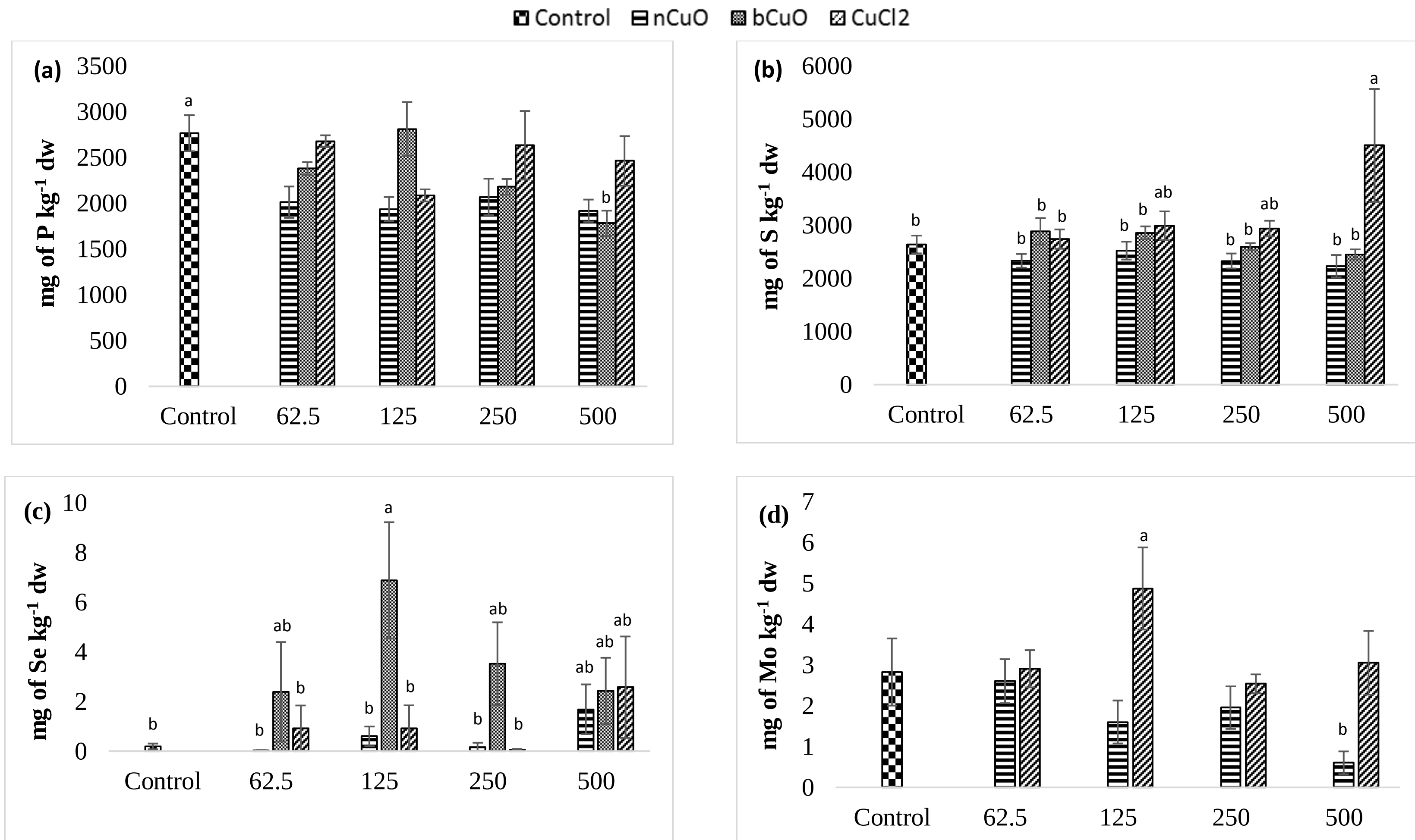


Figure 2.4: Concentrations of (a) P, (b) S, (c) Se, and (d) Mo in root samples of bell pepper plants cultivated for 90 days in natural soil amended with nCuO, bCuO, and $CuCl_2$ at 0 (control)-500 mg of Cu/kg of soil. Data are averages of four replicates ± SE. Averages with a common alphabet are not significantly different with respect to each other, as per one-way ANOVA and Tukey test ($p \leq 0.05$).

Leaf and fruit section

The concentration of elements altered in leaf and fruit are shown in Figures 2.5 and 2.6 including the macro elements calcium, magnesium, phosphorus, and sulfur and the microelements nickel and zinc.

Calcium: The concentrations of Ca^{2+} in the fruit samples are shown in Figure 2.6b. As illustrated in the figure, no significant differences were observed at any of the treatment concentrations respect to control. However, the Cu compounds affected in different way the concentration of Ca and other elements in the aboveground plant parts. At 500 mg/kg, Ca^{2+} content in the fruit samples was found to be significantly higher (85%) in the ionic Cu, compared to nCuO treatment ($p \leq 0.05$). Copper apparently shares transporters with Ca^{2+} that fall under heavy metal ATPases family (Kudla et al., 2010). Lysosomal cystine transporter (LCT)-1 under ABC transporters was found to transport Ca^{2+} along with heavy metals in wheat plants (Williams et al., 2000). A study conducted to assess the interaction between Cu and Ca^{2+} in soil grown Norway Spruce seedlings had results contrary to ours. Ionic copper treated plants had lower Ca^{2+} content in root and bark of the plants compared to control (Österås & Greger, 2006). Also, ionic copper amended sand grown cucumber plants (Alaoui-Sossé et al., 2004) and Cu treated hydroponically grown lettuce (Trujillo-Reyes, 2014) had lower Ca^{2+} in the leaf compared to control. However, in the current study, ionic copper showed much Ca^{2+} in fruit, compared to nCuO, which suggest an effect of particles.

Magnesium: Accumulation of Mg^{2+} in leaves is shown in Figure 2.5c. Bulk CuO and ionic copper significantly increased leaf Mg. At 500 mg/kg bCuO and ionic copper treatments, leaf Mg was elevated by 30% and 26 %, compared with control ($p \leq 0.05$). No response was observed from nCuO treatments. Alaoui-Sosse et al. (2004) and Trujillo-Reyes et al. (2014) found converse responses in sand grown cucumber and hydroponically grown lettuce. These researchers reported

lower Mg^{2+} in plant leaves, compared with controls. It has been reported that Mg^{2+} counters Cu toxicity in grapevine, wheat, barley, and cowpea by likely taking up the Cu binding sites (Juang et al., 2014, Guo et al., 2016). On the other hand, Cu treated oregano plants were found to have higher Mg^{2+} than control in the leaves, (Panou-Filotheou, 2001) coinciding with our results. Magnesium has a role in maintaining the permeability of cell membranes (Taiz & Zeiger, 2002). At high concentrations, Cu probably disturbed cell membranes and caused an influx of Mg^{2+} within the plant cells (Ochao et al., 2017).

The cation exchange capacity of the experimental soil was estimated to be between 15-30 meq/100 g, since it was silty loam, with a pH of 7.4. This cation exchange capacity could be affecting the uptake and translocation of Ca^{2+} as well as Mg^{2+} (http://extension.missouri.edu/p/MG4, accessed September 2017), under Cu treatment. Cu has a very high affinity for organic matter, clay, and sulfurous sites in soil or sediment (Flemming & Trevors, 1989, Lowry et al., 2012). Copper tends to replace the adsorption positions taken up by Ca^{2+} and Mg^{2+}, especially in organic matter, and releases the two ions in soil solution. This is apparently why there was (Figure 2.5c) significantly high leaf Mg at 500 mg/kg bCuO and ionic Cu treatments, compared with control. Figure 2.6b shows similar results with higher fruit Ca^{2+} at 500 mg/kg ionic Cu treatment, compared to nCuO.

Phosphorus: Phosphorus levels in leaf and fruit tissue of bell pepper plants are given in Figures 2.5a and 2.6a. As shown in the figures, leaf and fruit P contents were not significantly different respect to control ($p \leq 0.05$). However, at 125 mg/kg, the P concentration in leaf was lower for nCuO by 42%, compared with bCuO ($p \leq 0.05$) (Figure 2.5a). In fruit samples, the P concentration at 500 mg/kg nCuO was found to be significantly lower than bCuO at 62.5 and 125 mg/kg (33%, 35%) and lower than ionic Cu at 62.5 mg/kg (33%) ($p \leq 0.05$) (Figure 2.6a). Bulk CuO markedly

reduced the uptake of P in the root at the highest concentration, but it was apparently not that inhibitory at the lower concentrations, which enabled P translocation to leaf and fruit. Conversely, nCuO had a significantly inhibitory effect on P translocation up to the leaf and fruit. This was probably because of its aggregation characteristics (Karlsson et al., 2006, Lin et al., 2010, Lowry et al., 2012) and comparatively better Cu^{2+} ion release in soil solution compared to bCuO, as mentioned under the particle dissolution study (Figure 2.1). The finding for P in the leaf coincides with Hong et al. (2015) about P in hydroponically grown alfalfa shoots, where they saw a dose dependent increase in the P concentration in bCuO treated samples. The *Pht2* transporter is likely responsible for the translocation of P from the root up through the stem to leaf and fruit (Hong et al., 2015). Therefore, it seems that 125 mg/kg, bCuO and nCuO altered the gene expression for the P transporter; bCuO increased it, while nCuO reduced it.

Additionally, the ionic Cu treatment did not affect P uptake and translocation at any concentration. Most likely, the instant formation of Cu ions in solution in the ionic treatment initiated its binding with the available inorganic phosphate in soil. $Cu_3(PO_4)_2$ is an insoluble compound that occludes the available phosphate in alkaline soil (the pH of the experimental soil was 7.4, Table A2.5, Annex I) (Bardgett, 2005).

Sulfur: Concentrations of S in bell pepper leaf samples are shown in Figure 2.5b. As seen in this figure, ionic copper at 250 mg/kg increased S by 40%, respect to control and by 45% respect to 250 mg/kg nCuO treatment ($p \leq 0.05$) (Figure 2.5b). Ionic copper treatment was more responsive since the ionic form is more easily bioavailable to the plant (Eaton, 1942). At 250 mg/kg ionic Cu, S was significantly translocated to the leaf as against 500 mg/kg in the root. Sulfur was probably elevated at 250 mg/kg ionic Cu even at the root, but not significantly. Sulfate transporters *Sultr 2;1 and Sultr3;5* are responsible for sulfate translocation up the root to the aerial parts (Gigolashvili

& Kopriva, 2014, Gupta & Gupta, 2016). Shahbaz et al. (2013) observed an increased sulfate content in the shoot of Chinese cabbage plant at high ionic copper concentration under similar transporter effect. It could be possible that Cu ions bonded with sulfate in the soil and got translocated as $CuSO_4$ from the root to the leaves, thus, elevating the S content.

Selenium: Se concentrations in leaf and fruit samples are shown in Figures 2.5d and 2.6c. There were no significant differences observed at any of the treatments in leaf Se concentrations, compared with control. However, there were significant differences between treatments. At 125 mg/kg, Se from bCuO treatment was significantly higher by 431%, respect to that from nCuO ($p \leq 0.05$) (Figure 2.5d). On the other hand, there were no significant responses from ionic Cu or nCuO treatments individually, both in leaf and fruit. Though, at 250 mg/kg, fruit Se in bCuO treatment was higher by 1570% respect to the corresponding nCuO treatment and was higher than the control by 731% ($p \leq 0.05$) (Figure 2.6c). As mentioned before, bCuO has a larger negativity, compared with the other two treatments (Table 2.1). The negatively charged soil could be repelling the compound promoting its uptake and translocation through the plant (Lin et al., 2010), thus affecting Se translocation. *Sultr1* transporter is apparently responsible for the translocation of the absorbed selenate ions from the root through the stem to the leaves and to the fruit (Gupta & Gupta, 2016).

Nickel: The fruit Ni concentrations at different treatments are shown in Figure 2.6e. The concentrations of Ni^{2+} at 62.5, 125, 250 mg/kg bCuO treatments were significantly higher than the control by 159%, 136%, and 202%, respectively ($p \leq 0.05$). There were no changes observed in fruit Ni^{2+} concentration under nCuO and ionic Cu treatments individually. An important observation was made at 125 mg/kg, where the level of nickel in the fruit samples was significantly higher in bCuO treatment than in nCuO by 95% ($p \leq 0.05$) (Figure 2.6e). Cataldo et al.(1978)

pointed out that Ni^{2+} tends to form organic complexes once in the plant system, it is carried through the xylem and deposited in the leaf and the seed of soybean plant. This coincides with our finding where the presence of Ni^{2+} was observed in the fruit only.

Zinc: Concentrations of Zn in leaf and fruit samples are shown in Figures 2.5e and 2.6d. A distinct antagonistic effect between Zn and Cu was observed in fruit and leaf samples. Zn content in the leaf samples at 500 mg/kg nCuO was found to be significantly lower than control (55 %), bCuO treatments at 62.5 and 125 mg/kg (52% each), and lower than ionic Cu at 62.5 mg/kg (55%) ($p \leq 0.05$) (Figure 2.5e). Moreover, in the fruit samples Zn levels at 500 mg/kg nCuO treatment were significantly lower than control (47 %), lower than 62.5, 125, 250 mg/kg bCuO (54 %, 48 %, 51 %) and lower than 62.5 mg/kg ionic Cu treatment (44 %) ($p \leq 0.05$). (Figure 2.6d). Once again, we observed a nutrient inhibitory effect under nCuO exposure and a nutrient promoting effect under bCuO and ionic Cu exposure. These observations in shoots were in agreement with studies conducted by Kabata-Pendias and Pendias (1984), Lastra et al. (1987), Beckett and Davis (1978), and Safaya (1976). In a study conducted to assess the fertilization qualities of Zn with different rice varieties, it was observed that Cu and Zn had an antagonistic effect on each other, especially at the primary absorption site, negatively affecting its translocation (Cayton et al., 1985). Cu treatment has been found to reduce the Zn uptake in some other plant studies with cereals, wheat, and rice (Chaudhry & Loneragan, 1972, Rashid et al., 1976). Zuverza-Mena et al. (2015) reported that Zn was hampered under a high nCuO concentrations in cilantro shoot.

The recommended dietary intake for Zn, according to the Food and Nutrition Board, Institute of Medicine (Dietary Reference), is 8-11 mg/day. For the fruit data, the lowest observed value under the treatments was 7 mg/kg (Figure 2.6d). This could indicate a low Zn content;

however, it is premature to assure that the reduction in fruit Zn compromises bell pepper nutritional quality.

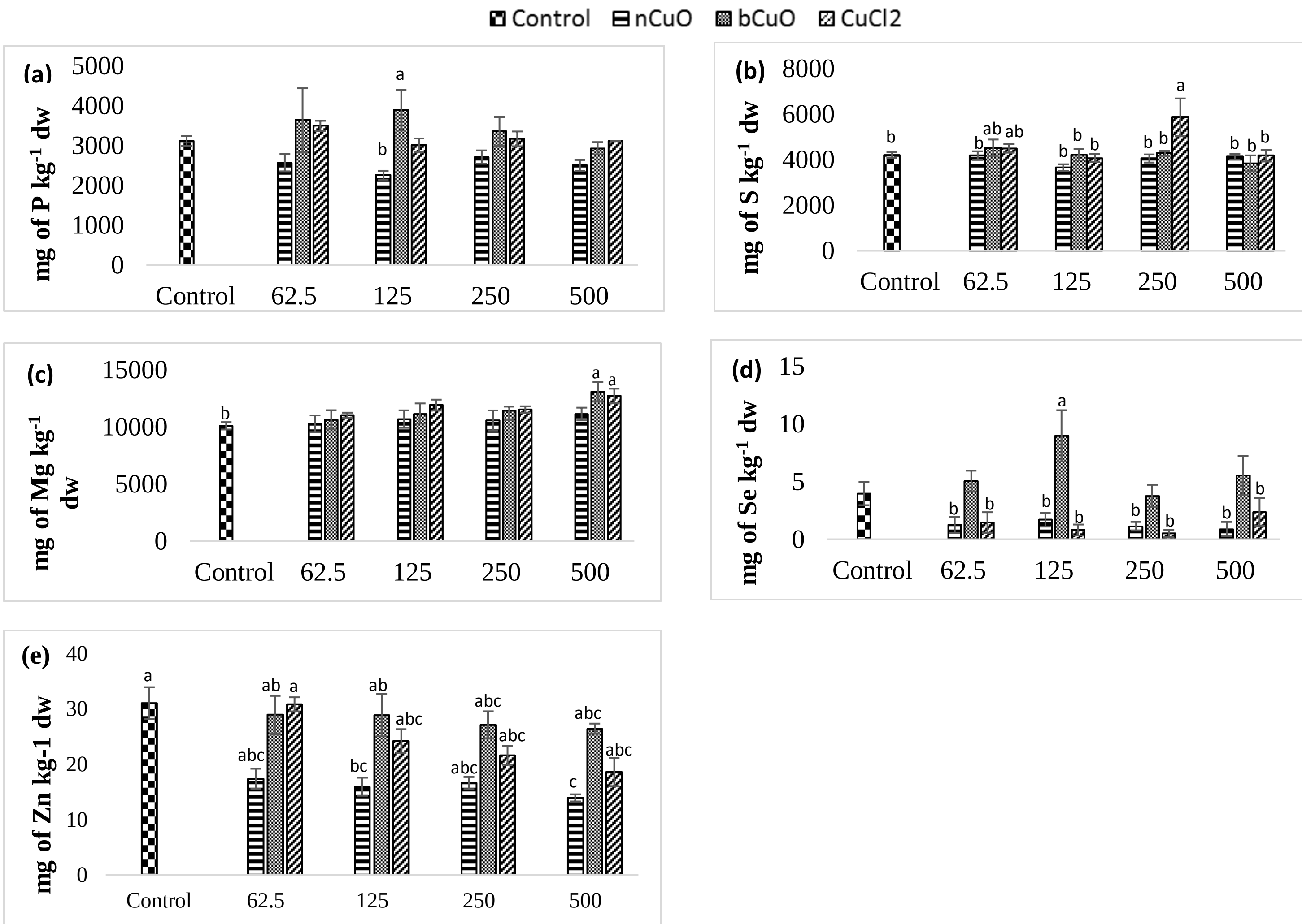


Figure 2.5: Concentrations of (a) P, (b) S, (c) Mg, (d) Se, and (e) Zn in leaf samples of bell pepper plants cultivated for 90 days in natural soil amended with nCuO, bCuO, and $CuCl_2$ at 0 (control)-500 mg of Cu/kg of soil. Data are averages of four replicates ± SE. Averages with a common alphabet are not significantly different with respect to each other, as per one-way ANOVA and Tukey test ($p \leq 0.05$).

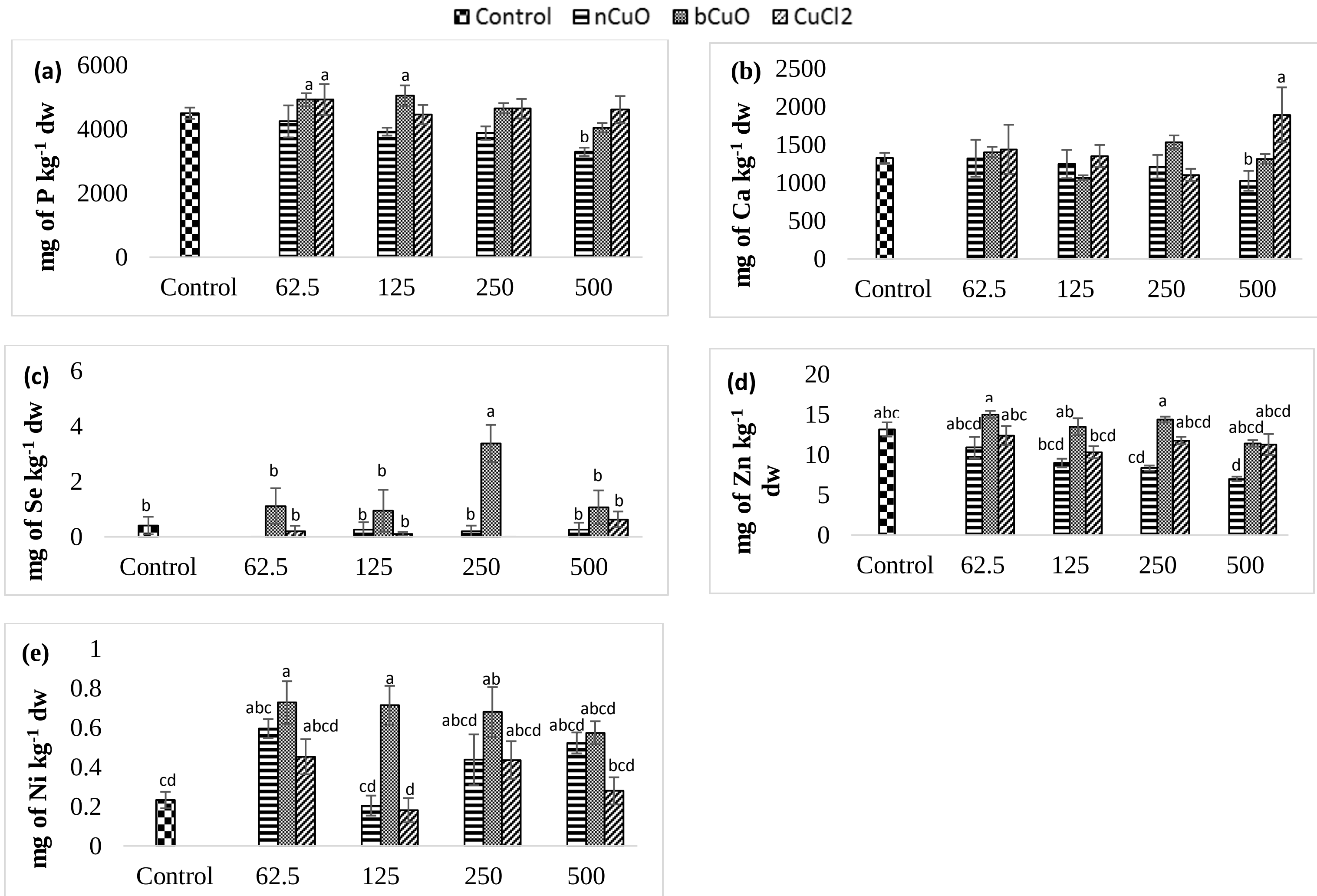


Figure 2.6: Concentrations of (a) P, (b) Ca, (c) Se, (d) Zn, and (e) Ni in fruit samples of bell pepper plants cultivated for 90 days in natural soil amended with nCuO, bCuO, and $CuCl_2$ at 0 (control)-500 mg of Cu/kg of soil. Data are averages of four replicates ± SE. Averages with a common alphabet are not significantly different with respect to each other, as per one-way ANOVA and Tukey test ($p \leq 0.05$)

2.3.6 Effect of treatments in fruit production

The total mass of the fruit collected per plant, average weight of fruit per plant, the total number of fruit collected per plant and their averages per treatment were determined and analyzed. None of the fruit production parameters showed a statistically significant difference at any concentration ($p \leq 0.05$).

2.3.7 Dry biomass production and foliar area

The root, stem, and leaf dry biomass data are shown in Annex I (Figure A2.1). There were no statistically significant differences in the dry biomass collected and the foliar area (Figure A2.2, Annex I) observed from the different treatments ($p \leq 0.05$).

2.4 Conclusions

Although none of the treatments affected leaf chlorophyll, the gas exchange results showed that the ionic copper treatment had a significant deteriorating effect on evapotranspiration, photosynthesis, and stomatal conductance. As in the previous studies, Cu tended to remain in bell pepper roots, independent of the compound; however, there were variable effects on nutrient accumulation. Bulk CuO treatment bolstered the nutrient uptake and translocation at certain concentrations of most elements in the plant system, while nCuO mostly had an inhibitory effect. Interestingly, under ionic Cu treatment, the plant response to nutrient uptake was almost the same as that under bCuO treatment. The plant biomass and fruit production data did not show any significant variations under the three treatments. With exception of nCuO at 500 mg/kg that reduced Zn, compared with control, none of the concentrations reduced the absorption of essential elements in fruit. Overall, nCuO had inhibitory effect on the uptake of important elements but did not show visible signs of toxicity or effects in plant productivity.

Chapter 3: Differential physiological and biochemical effects of nano *vs.* micron Cu at two phenological growth stages of bell pepper (*Capsicum annuum*) plants

3.1 Introduction

The prospective use of engineered nanoparticles (ENPs) [tiny particles with at least two dimensions ≤ 100 nanometers (10^{-9} meters)] for the smart delivery of nutrient elements to plants began in 1990s (Gogos et al., 2012). As time passed, ENPs have shown other agricultural applications. Copper-based nano products have a considerable market in agriculture because they are used as fertilizers and pesticides, or in electronics and sensors that support precision agriculture (Liu & Lal, 2015, Dubey & Mailapalli, 2016, Pandey et al., 2016). Pertinent literature has shown that, besides the beneficial effects, ENPs have been shown to produce toxicity symptoms. Regarding the toxicological aspect, dose and growth environment determine the beneficial or detrimental effects of ENPs (Reddy et al., 2016). Regulations regarding the ENPs' production and use are still in their formative stages (Ellenbecker & Tsai, 2015). Hence, given the increasing market of nano-enabled products, studies on their ecological/physiological effects and risk assessment are highly imperative.

The database of plants and Cu ENPs' interaction studies is continually increasing. Trujillo-Reyes et al. (2014) found a negative effect on root length, dry biomass, and moisture content on hydroponically grown lettuce plants exposed to 10 and 20 mg nCu/L. According to Zuverza-Mena et al. (2015), seed germination in cilantro plants was hampered at 20 mg nCu/kg and 80 mg bCu/kg treatments. Soil borne 80 mg nCu/kg significantly reduced cilantro shoot length, at maturity, compared to control. A metabolomics study in hydroponically grown cucumber plants showed that nCu (10 and 20 mg/L) interfered with root nutrient uptake (Zhao et al., 2016). These researchers

noticed stress related metabolic activity in root exudates. The root activity included up-regulation of phenolic compounds, amino acids, and ascorbic acid, and downregulation of citric acid, which are used by plants as a defense mechanism against metal toxicity (Zhao et al., 2016). Subsequently, Zhao et al. (2016b) cultivated cucumber plants in soil amended with 200, 400, and 800 mg nCu/kg, finding an altered metabolites profile in the fruit under the two higher treatments. At such concentrations, nCu up-regulated proline, glycine, valine, xylose, and benzoic acid, while down-regulated citric acid, 1-kestose, myo-inositol, and ornithine. Du et al. (2018) exposed oregano plants to nCu and bCu through soil media and found bCu to adversely affect the root biomass, root length, and water content. Ogunkunle et al. (2018) examined the effect of 0-1000 mg nCu/kg on cowpea plants and found an altered enzyme activity, as well as 250% higher Cu in the seeds, compared to control. Beneficial effects of nCu have also been reported. The root application of biosynthesized nCu (2.5 mg/kg) in tea plants infested with red root-rot disease (*Poria hypolateritia*) resulted in higher leaf area and 52.7% disease reduction (Ponmurugan et al., 2016). The above literature suggests that nCu effects are largely dependent on the dose, exposure mode, plant species, and growth conditions. Extensive information of comparative effects of particle size (bulk vs nano) of metal based compounds on plant growth and proliferation characteristics are given by Rawat et al. (2017).

Bell pepper (*Capsicum annuum* L.) was selected as the model plant to study the effects of long exposure time to metallic copper. As in the previous study, natural soil was selected as the plant growth matrix. This work is an extension of the previous work about the physiological effects of nCuO, bulk CuO, and ionic Cu on full life cycle of bell pepper plants (Rawat et al., 2018). The study at hand is a similar analysis of biochemical and physiological effects of nCu vs bCu on the same plant species, the same natural soil and under the same growth conditions. Additionally, the

study compares the effects of the two copper species at the vegetative stage (45 days/half life cycle) vs the reproductive stage (90 days/maturity) of the plant life cycle. Comparison of the half vs full life cycle makes this study unique and an add-on to existing body of work of ENPs interaction with soil and plants.

3.2 Method and material

3.2.1 Characterization of the copper-based chemicals

Nano-particulate copper (nCu) was procured from US Research Nanomaterials (Houston, TX) and bulk copper (bCu) from Sigma Aldrich (St. Louis, MO). The characterization of the chemicals is as given on Table A3.1, Annex II (Hong et al., 2015, Lin et al., 2015).

3.2.2 Soil characterization and contaminant treatments

The plants were grown in agricultural soil collected at a cotton field in Socorro, TX (latitude: N 31°41' 30.705” and longitude: W 106°17' 13.936”, elevation: 1,115 m asl). The soil characterization has been previously done and described as silty loam (65% silt, 20% sand, and 15% clay) (Ryżak et al., 2011, Rawat et al., 2018). The soil pH, total dissolved solids, and electrical conductivity in DI water, as determined by Hanah™, are given on Table A3.2 (Annex II). The soil moisture and organic matter content were determined by loss on ignition method (Ben Moshe et al., 2013).

Bell pepper seeds (California wonder 300 TMR) were planted in starting mix and 30 day old seedlings were transplanted into pots with amended soil. For the vegetative stage (VS) study, 2 kg capacity pots were prepared for transplantation by amending the soil with nCu and bCu at 0 (control), 62.5 and 500 mg/kg treatments, three replicates each and one plant per pot. For the reproductive stage (RS) study, pots were prepared similarly with nCu and bCu at 0 (control), 62.5, 125, and 500 mg of Cu/kg of soil, three replicates each, 5 kg soil in each pot, and one plant per pot. The soil was homogenized with an aqueous suspension of the copper chemicals, prepared by

stirring in DI water for 30 min. The copper chemicals were suspended in enough water to have the soil at about 60% of the field capacity. While preparing the substrate, contaminant homogeneity was ensured by thorough manual mixing of the soil with the chemical suspension for 20 min.

The Cu concentrations were selected based on the environmental and amendment concentrations. According to USGS data, the average Cu concentration in different kinds of soil is 30 mg/kg dry weight (https://pubs.usgs.gov/pp/1270/pdf/PP1270_508.pdf, accessed September 2017). Among agricultural amendments, pesticides have about 50 mg Cu/kg, fertilizers about 300 mg Cu/kg, and sludge has about 3000 mg Cu/kg. The Cu concentrations in fly ash range from 300 mg/kg to about 2000 mg/kg (https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=206&tid=37, accessed September 2017). The concentration range selected for this study is most environmentally relevant to agricultural fields. Additionally, this study is trying to follow suit from previous studies.

3.2.3 Copper dissolution in soil solution

The analysis for Cu concentration in the soil solution was performed as described in the previous study (Rawat et al., 2018). To summarize, Cu concentration in soil solution was determined for nCu and bCu species. Three replicates each with 20 g of the experimental soil mixed with the chemical and 30 mL DI water to make 500 mg/kg aqueous suspension were shaken for 12, 24, 36, and 48 h. The supernatant soil solutions were collected and centrifuged, the process being repeated 3 times to exclude any NPs in suspension. The copper ion concentration was determined via ICP-OES analysis. The detailed method is described in our previous publication with a similar experiment (Rawat et al., 2018).

Standard reference material 1570a from the National Institute of Standards and Technology was used to evaluate the extraction efficiency of the system, obtaining a recovery of 101.11 ± 5.59%. For quality control of the ICP readings, blank and a spiked sample containing Cu 20 mg/L were read every 25 samples. The average readings for Cu in the spiked sample was 21.30 ± 0.58

mg/L, thus validating the digestion process. The ICP-OES parameters used were as follows: nebulizer flow, 0.80 L/min; power, 1,400 W; peristaltic pump rate, 1.5 mL/min; flush time, 20 s; delay time, 20 s; read time, 10 s; and wash time, 60 s.

3.2.4 Plant culture at the greenhouse

Growing of seeds to seedlings, their transplantation to Cu amended potted soil, plant growth and maturity, and harvest were conducted at the greenhouse. The plants were cultured for 45 days till full vertical growth and budding, for the VS and until 90 days till maturity (fruiting), for the RS. Details of the procedures and conditions at the green house are given in the previous publication (Rawat et al., 2018). Throughout the experiment, the average day temperature at the green house was 30.1 ± 3.6 ˚C and the average night temperature 26.6 ± 3.5 ˚C. The average daylight was 12.1 ± 5.1 $mol \cdot m^{-2} \cdot d^{-1}$, while the relative humidity was 40.4 ± 14.6%.

3.2.5 Gas exchange and chlorophyll measurement (SPAD)

Photosynthetic rate, stomatal conductance, and evapotranspiration are the three markers considered as a measure of gas exchange. This data was collected in the treated plants at the end of the life cycle before the harvest using CIRAS-3 portable photosynthesis system (Amesbury, MA). The instrument probe contains a leaf cuvette used to hold the newest fully expanded leaf of each plant and take the measurement. The plants were kept well-hydrated before measurements taken between 1000 to 1400 h on sunny days. Any cloud cover would confound the readings (Zhao et al., 2013). In addition, relative chlorophyll content was determined using a hand held single photon avalanche diode chlorophyll meter (SPAD, Minolta Camera, Japan) by taking the average value across ten leaves per plant.

3.2.6 Enzyme analysis

Leaf and root fresh tissue extracts were prepared in 25 mM phosphate (KH_2PO_4) buffer (pH: 7.4) for catalase (CAT), and 0.1 M phosphate buffer (pH: 7.4) for ascorbate peroxidase

(APOX) analysis. These extracts were run to measure enzyme kinetic activity by UV-visible spectrophotometry (Gallego et al., 1996, Murgia et al., 2004).

3.2.7 Vitamin C analysis

Vitamin C content in the bell pepper fruits was determined by a titration method on the fruit juice using 2, 6-dichloroindophenol dye as the titrant. Ascorbic acid was used as the standard and a mixture of metaphosphoric acid and acetic acid was used as the analyte along with the fruit juice. This is an official method used as a rapid QC for some commercial products (AOAC Method 967.21, Eitenmiller & Landen, 2003).

3.2.8 Total sugars determination

Total sugar extracts were prepared following Verma and Dubey (2001) prescribed method. Ethanolic sugar extract was obtained from dried plant tissue. The analysis of total sugar was performed by measuring the color change in phenol-H_2SO_4 complex on a microplate format (Masuko et al., 2005). Glucose was used as standard for the analysis. The microplate spectrophotometer employed for the assay was SPECTRAmax190, Molecular Devices, Sunnyvale, CA.

3.2.9 Pigment content (carotenoid, chlorophyll a and b) analysis

Bell pepper fruit extracts were prepared with 80% acetone and the pigments were estimated by measuring absorbance on a plate reader. The method used was as suggested by Lichtenthaler and Wellburn (1983). The plate reader used was SPECTRAmax190, Molecular Devices, Sunnyvale, CA.

3.2.10 Harvest and elemental analysis

Forty five (45) days post transplantation for the VS and ninety (90) days post transplantation for the RS, the plants were growing and budding, or were flowering and fruiting. The total foliar area, total number of leaves, flowers, fruits and their respective total weights were

recorded for each plant. The total foliar area was measured by LI-3100C area meter (LI-COR Biosciences, Lincoln, Nebraska, USA). The separated samples were washed in 0.01M HNO_3 followed by DI water. The stem and root tissues were also collected separately, cleaned, weighed, measured, and dried. After air drying, the fresh samples were oven dried at 60-70˚C for 72 h.

The dried plant samples were weighed to determine the dry biomass. Samples were ground and the powders were digested in a digiPrep hot block (SCP Science, Quebec, Canada) for further analysis. The Environmental Protection Agency (EPA) method 3051 was employed for digestion using plasma pure nitric acid (65%) and hydrogen peroxide (30%) (1:4). Inductively coupled plasma-optical emission spectroscopy (ICP-OES, Perkin-Elmer Optima 4300 DV, Shelton, CT) was used to determine the macro and micro elements (Ca, K, Mg, P, S, Fe, Zn, Cu, Mn, Ni, Mo) concentrations in the digested samples (Zuverza-Mena et al., 2015). Three blank sample carriers, two carriers with spinach as the standard reference material (NIST 1570a, Gaithersburg, MD) and three with samples spiked with 20 mg/L of the Cu standards were all digested and included in the analytical process as part of the QA/QC (Hong et al., 2015).

3.3 Results and discussion

3.3.1 Soil dissolution study

Figure 3.1 shows that at 500 mg/kg, nCu went more easily into solution, compared to bCu, yielding much higher Cu concentrations in soil solution. The Cu ion concentration was higher by 478.33% at 12 h, 287.61% at 24 h, 237.42% at 36 h, and 153.12% at 48 h for 500 mg nCu/kg, compared to 500 mg bCu/kg treatments ($p \leq 0.05$). In addition, Cu ion concentration for 500 mg nCu/kg was higher by 33.36% at 36 h, compared to that at 12 h ($p \leq 0.05$). Similarly, for 500 mg bCu/kg, the Cu ion concentration was higher by 166.97% at 48 h, compared to that at 12 h of dissolution ($p \leq 0.05$). The trends were similar to those observed in our previous study with, nano and bulk CuO at 500 mg/kg in the same soil (Rawat et al., 2018). Additionally, Du et al. (2018)

conducted Cu fractionation in soil and obtained 387.5%, 50.0%, and 303.7% higher Cu concentration from nCu than bCu at 50, 100, and 200 mg/kg, respectively. This agrees with McShane et al.'s (2014) finding with nano and bulk CuO in sandy loam soils. However, our findings do not align with those observed by Dimkpa et al. (2012) with nCuO in sand matrix, where they saw no significant difference in the nano and bulk CuO dissolution, except at 0 days (p=0.05). This could be due to differences in matrix (sand *vs.* soil) and in the Cu species (CuO *vs.* metallic Cu). In addition, critical media characteristics like organic matter and pH determine the ENP fate and dissolution. Sand matrix used by Dimkpa et al. (2012) (pH: 7.9) contained negligible OM, whereas the soil used in this study (silty loam, pH: 7.4) had 2% OM (Table A3.2, Annex II). In addition, the incubation time (14 days *vs.* 48 h) and shaking (intermittent *vs.* continuous) were different in the two studies and could have affected the dissolution kinetics.

Figure 3.1 also shows that the Cu ion concentration in the soil solution, at 500 mg nCu/kg, increased gradually with time. Sekine et al. (2017) attribute this fact to soil alkalinity (pH 7.4), where dissolution of nCu starts slowly and increases with time. This idea is supported by McShane et al. (2014) and Gao et al. (2017). We also noted on Table A3.1 (Annex II) that bCu size was about an order of magnitude bigger than that of nCu. A smaller particle size and hydrodynamic diameter provide a larger total surface for interaction with media; hence, more dissolution over time (Bawa et al., 2016).

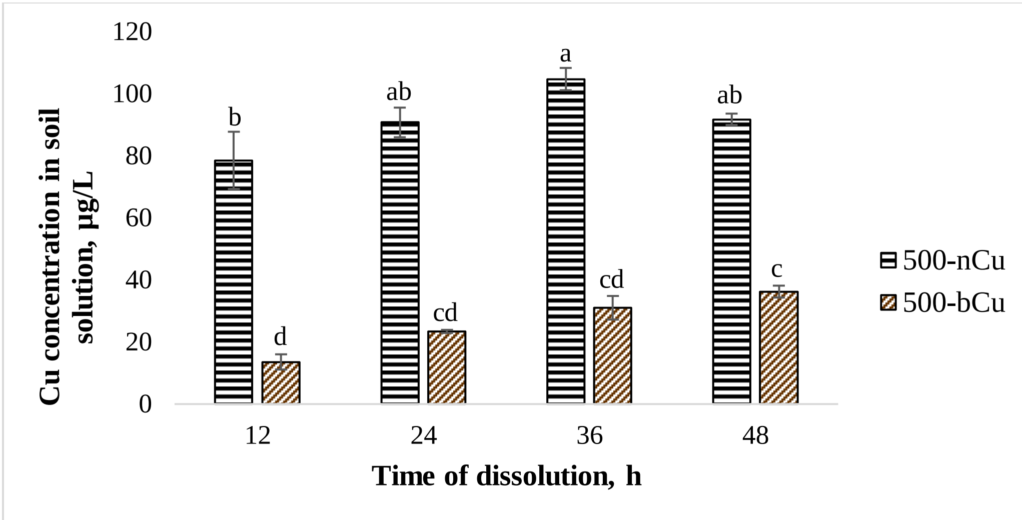


Figure 3.1: Relative dissolution of copper in soil solution in DI water, minus the pristine copper present in natural soil. Data are averages of three replicates ± SE. Averages without a common letter are significantly different with respect to each other, as per one-way ANOVA and the Tukey test ($p \leq 0.05$).

3.3.2 Effect of treatments on root growth, Cu uptake, and bioaccumulation

Vegetative stage study (VS)

Figure 3.2 shows effects of the Cu treatments on plant roots at the vegetative stage (VS). As given on Figure 3.2a, at 500 mg nCu/kg, the root dry weight was higher than control by 187.18% ($p \leq 0.1$). Figure 3.2b shows that the root length, at the same treatment, was higher by 58.46%, compared to control ($p \leq 0.1$). Additionally, root fresh weight at 500 mg nCu/kg was higher by 142.26%, compared to 62.5 mg/kg nCu treated plants ($p \leq 0.1$, Figure 3.2c). This indicates a fertilizing and growth stimulatory effect of nCu treatment on bell pepper root at the VS. Musante and White (2012) exposed *Cucurbita pepo* to nCu and bCu for 14 days in hydroponics and found nCu to reduce the plant biomass more than bCu, and show toxicity. The growth media and plant species differences account for the antagonistic findings. Notwithstanding, our results agree with Hafeez et al.'s (2015) finding, where they observed nCu (10-50 mg/kg) to promote growth and yield in soil grown wheat plants. The gas exchange and chlorophyll content measured in the leaves at VS, in the current study, showed no adverse effects (Figure A3.1, Annex II) as did the agronomical parameters in the aerial parts of the plant. It is being hypothesized that most of the nCu added to the soil was aggregated (given the small size) in the rhizosphere during the VS, affecting the root growth. The soil pH of 7.4 and organic matter content of about 2% support the statement (Table A3.2, Annex II). Both homo and hetero-aggregation are a possibility under the given conditions (Hotze et al., 2010). Nano Cu could also have been adsorbed on the root surface post aggregation, which could have made the ionization further slow and constricted (Zhou et al., 2011). It probably ionized gradually with time and was translocated to the aerial parts of the plant more in the RS (McShane et al., 2014, Gao et al., 2017).

As seen in Figure 3.3, Cu ions from only bCu treatments were taken up and transported significantly in the root and aerial parts of the plants in the VS. Micron sized (bulk) Cu likely had

negligible aggregation properties (Du et al., 2018). Taking a look at Table A3.1 (Annex II) for the zeta potential of the two Cu species, we notice that the zeta potential for bCu (-35.4±1.27) is beyond the coagulation limit (≤ ± 30mV), unlike nCu (-29.4 ± 0.8) (Rawat et al., 2018). However, this finding does not align with the results on Figure 3.1, probably because the experiment with plants grown in amended soil did not include the reverberations, unlike the soil dissolution study (Bandopadhyay et al., 2015, Mukherjee et al., 2015). The plant based experiment also spanned a much longer time, compared to soil dissolution study (45/90 days *vs.* 4 days). Additionally, the presence of a living plant root in the rhizosphere could have made a difference between the two types of analyses. Plant root exudates, like organic acids, could also have an effect on the nCu/bCu dissolution and bioavailability in the plant experiment (Zhao et al., 2016).

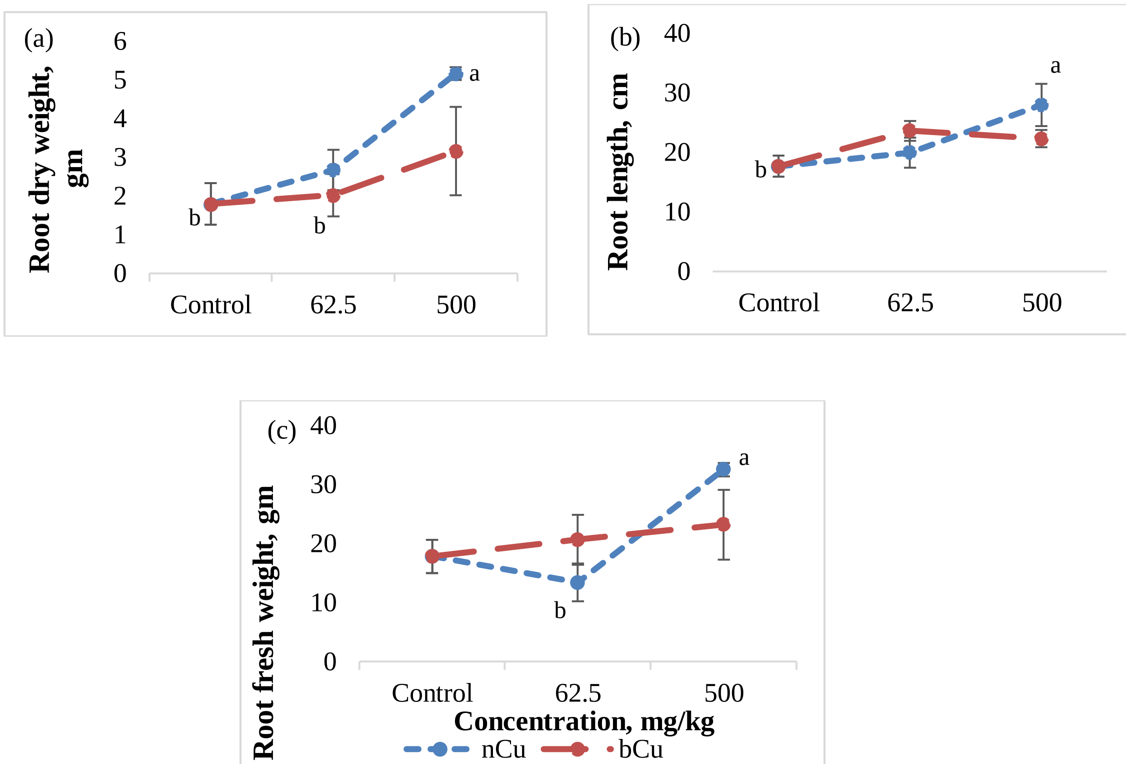


Figure 3.2: Root growth statistics (a) root dry weight (b) root length (c) root fresh weight in the bell pepper plants cultivated under nCu and bCu amended soil at 0 (control), 62.5, and 500 mg of Cu/kg of soil for 45 days. Data are averages of three replicates ± SE. Averages without a common letter are significantly different with respect to each other, per one way ANOVA followed by Tukey test ($p \leq 0.1$).

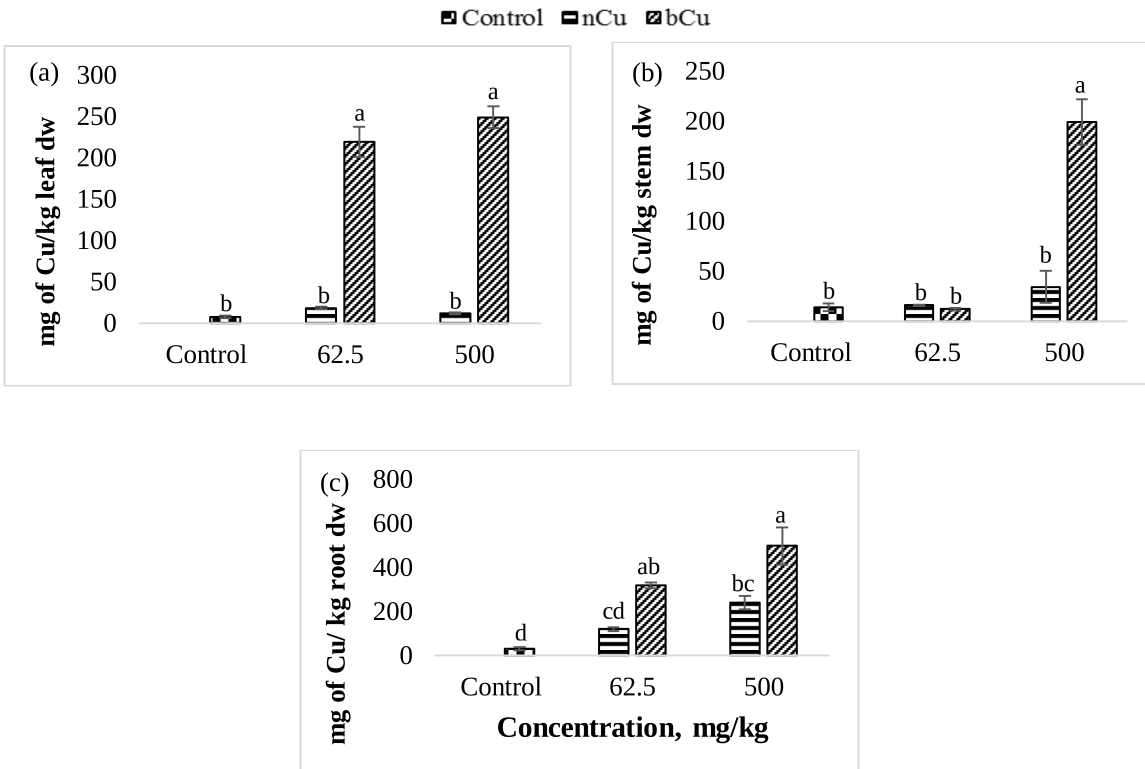


Figure 3.3: Cu concentrations in the (a) leaf (b) stem and (c) root for the vegetative stage study (VS) in the bell pepper plants cultivated under nCu and bCu amended soil for 45 days at 0, 62.5, and 500 mg of Cu/kg of soil. Data are averages of three replicates ± SE. Averages without a common letter are significantly different with respect to each other, as per one-way ANOVA followed by Tukey test ($p \leq 0.05$).

Reproductive stage study (RS)

For the reproductive stage study (RS), 500 mg nCu/kg yielded 121.17%, while 500 mg bCu/kg yielded 83.62% higher root Cu, compared to control ($p \leq 0.05$) (Figure 3.4b). The leaf Cu content at 500 mg bCu/kg treatment was lower than control by 92.65% and lower than that at 500 mg nCu/kg treatment by 93.79%. ($p \leq 0.05$) (Figure 3.4a). These results are converse to that of Zuverza-Mena et al. (2015) in soil grown cilantro plants, who reported no significant differences in root Cu. They observed significantly higher Cu bioaccumulation in the shoot for both nCu and bCu treatments, compared to control. But there were no significant differences between nCu and bCu outcomes. Their plant species and the exposure periods (30 vs 90 days) were different from the current study. Trujillo-Reyes et al. (2014), hydroponically exposed lettuce plants to self-synthesized core –shell Cu/CuO nanoparticles at 10 and 20 mg/L for 15 days. They saw significantly high Cu bioaccumulation in the root at 10 and 20 mg/L and in the leaves at 20 mg/L, compared to control. Their finding agrees with our results, although the growth media and plant species were different. Servin et al. (2017) exposed lettuce plants to freshly applied nano and bulk CuO at 400 mg Cu/kg soil for seventy (70) days. They also observed significantly higher root Cu bioaccumulation in both the treatments, compared to control ($p \leq 0.05$).

Contrary to VS, copper from bulk Cu treatments was not significantly detected in the aerial parts of the plant during RS. It was probably metabolized into other organic forms past the 45 day mark, plant moving from vegetative to reproductive stage. It could also have been translocated to the bell pepper fruit enabling its growth and maturity. Different cellular processes counteract the metal uptake in the plant as it matures (45-90 days). Some of these processes are the production of reactive oxygen species (ROS), sequestration in metal tolerant enzymes, and formation of complexes like phytochelatin, metallothioneins, and sulphur-coordinated complexes. Formation

of acidic metabolites like citrate, proline, or histidine enable chelation of excess Cu. In addition, excess Cu sequestration into metabolically inactive cell organelles such as cell wall, vacuole and apoplast, and accumulation of secondary metabolites could be going on in the plant system (Fernandes & Henriques, 1991, Adrees et al., 2015). It was also observed that at 90 days, the catalase and ascorbate peroxidase enzymes in roots and leaves did not show any effect of the Cu treatments (Figure A3.2, Annex II). The effect was probably countered by the plant by the stated mechanisms, as it matured. Additionally, fruit nutrient quality in terms of chlorophyll, carotenoid (vitamin A), sugar content, and vitamin C were least affected by any of the Cu treatments (Figure A3.4, Table A3.4, Table A3.5, Figure A3.3, Annex II). This suggests that the fruit development in bell pepper is not a sink for excess Cu and other organic compounds. A higher concentration of the treatments (> 500 mg/kg) could have been translocated to the fruit and probably had an effect.

It is hypothesized that Cu, from nCu treatments, was better translocated over the span of 90 days of soil exposure, apparently because of better dissolution (Figure 3.1), possible aggregation, root adsorption, and consequently, slow and gradual ionization in the rhizosphere (McShane et al., 2014, Gao et al., 2017). Adrees et al. (2015) noted that only 1-20% of the soil Cu is bioavailable to plants, while most of it gets bound to the organic matter (2% OM in the current study). The trends seen in Figure 3.4 relate to the amended Cu in addition to the natural soil Cu content.

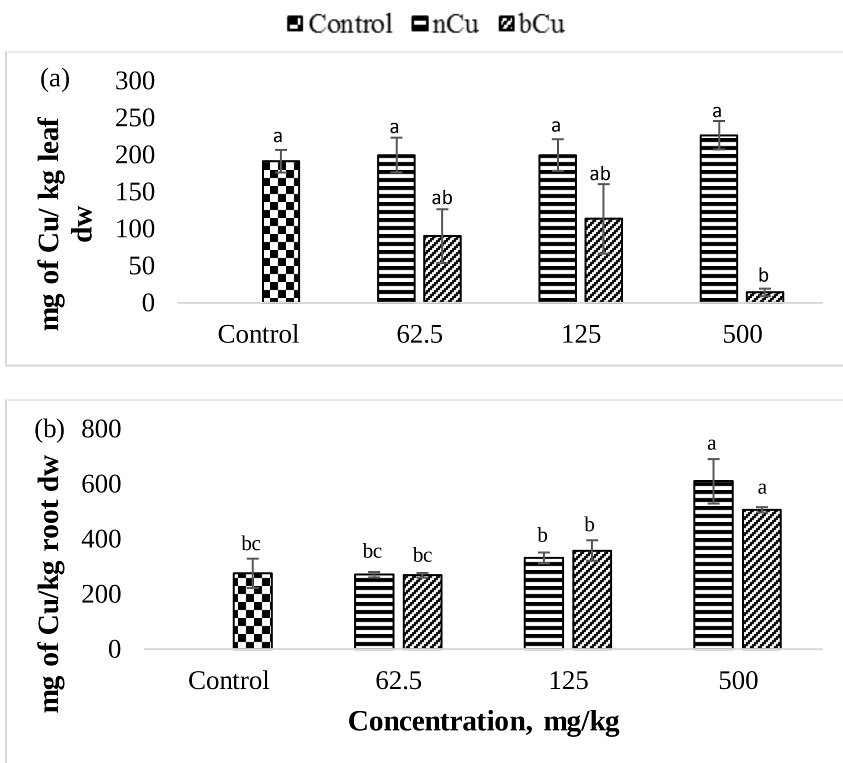


Figure 3.4: Cu concentrations in the (a) leaf (b) root in the bell pepper plants cultivated under nCu and bCu amended soil for 90 days at 0, 62.5, 125, and 500 mg of Cu/kg of soil. Data are averages of three replicates ± SE. Averages without a common letter are significantly different with respect to each other, as per one way ANOVA followed by Tukey test ($p \leq 0.05$).

3.3.3 Comparison between the vegetative stage (VS) vs. reproductive stage (RS) studies

Copper in the root and aerial parts of the plant

As shown in Figure 3.5, there was a stark difference in the Cu uptake and translocation between nCu/bCu treatments at VS and RS. The concentration of Cu in leaves was significantly higher for nCu treatments in RS, compared with VS, 985.3% at 62.5 mg/kg and 1738.8% at 500 mg/kg treatments ($p \leq 0.05$, Figure 3.5a). Conversely, leaf Cu concentration in bCu treatments was significantly higher during VS, as against RS, 1516.6% higher at 500 mg/kg treatment ($p \leq 0.05$). This finding aligns with what Majumdar et al. (2014) saw in hydroponically grown kidney bean plants under $nCeO_2$ exposure. Moving from 1 day to 7 days to 15 days of exposure, the Ce concentration in the farther aerial tissues (leaves) increased significantly over time. Further molecular level studies are needed to understand the exact reasons for the opposite uptake tendencies between the two exposure periods, and the different mechanisms of action of the two Cu species. There were, apparently, acute changes taking place in the cellular homeostasis between the vegetative and the reproductive stages that have affected the plant response to the Cu treatments (Adrees et al., 2015).

Looking at the comparative stacked bar chart on Figure 3.5c, the root Cu bioaccumulation at 500 mg nCu/kg, for RS, was 297.66 % and 153.95% higher than control and VS value at the same treatment concentration, respectively ($p \leq 0.05$). For stems, the Cu concentration from nCu treatments, in the RS, was 1064.92% higher at 62.5 mg/kg and 458.99% higher at 500 mg/kg than that at VS ($p \leq 0.05$, Figure 3.5b). As mentioned earlier, some studies support the fact that, in alkaline conditions, nCu dissolution in soil gradually increases with time (McShane et al., 2014, Gao et al., 2017). This apparently also applies for the Cu uptake in the root at 500 mg nCu /kg treatment. The translocation of Cu likely reflects the difference in nCu dissolution in soil during short term and long term (Figure 3.5).

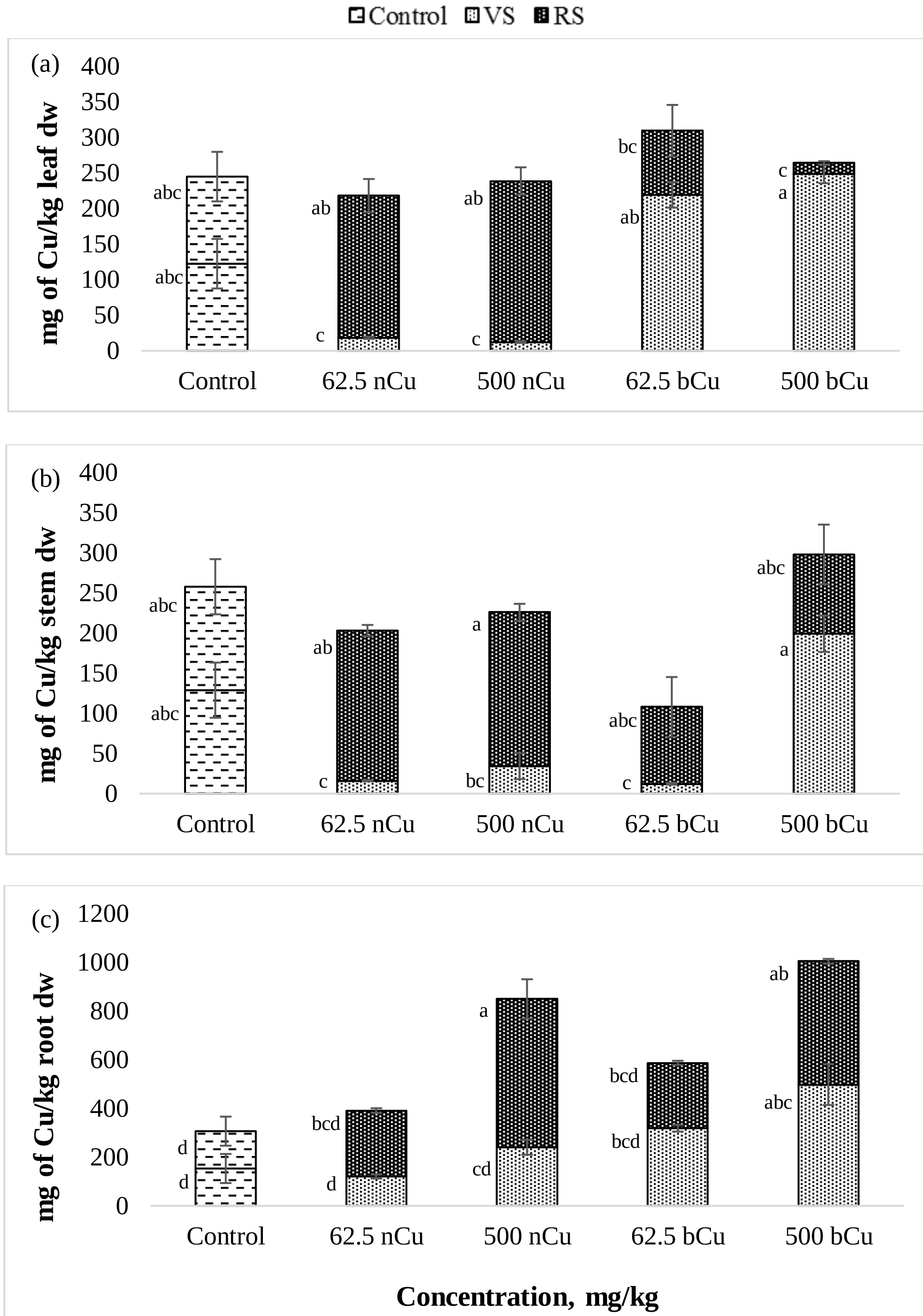


Figure 3.5: Comparison of the Cu concentration in (a) leaf (b) stem and (c) root tissue at vegetative vs reproductive stage (45 vs 90 days) study in bell pepper plants cultivated under nCu and bCu treatments at 0, 62.5, and 500 mg of Cu/kg soil. Data are averages of three replicates ± SE. Averages without a common letter are significantly different with respect to each other, as per one-way ANOVA and Tukey test ($p \leq 0.05$).

3.3.4 Macro-elements in the flower/fruit

Some of the prominent differences in the macro-mineral nutrients between the fruit and flower stages have been elaborated here. These include comparison in calcium, magnesium, and sulfur concentrations in fruit and flower.

Calcium

Figure 3.6a shows that the Ca concentration in bell pepper flowers was significantly higher by 209.14% at 62.5 mg nCu/kg and by 214.965% at 62.5 mg bCu/kg treatment, compared to control ($p \leq 0.05$). Also, Ca was higher in flowers than in fruits by 1209.33% at 62.5 mg nCu/kg, by 822.88% at 62.5 mg bCu/kg, and by 594.58% at 500 mg bCu/kg ($p \leq 0.05$). Fruits are innately low in Ca, unlike the flowers (Hanger, 1979). Moving from flowering to fruiting stage, the supply of Ca from the roots does not match up the pace of rapid fruit growth and cell enlargement. Per supporting literature, excess of the hormone gibberellins causes a decline in Ca uptake in the fruit (Saure, 2005). Hence, we do not attribute the above findings to the Cu treatments.

Magnesium

As shown on Figure 3.6b, the Mg concentration in bell pepper flowers, at 62.5 mg nCu/kg, was higher than that in control by 102.95% ($p \leq 0.05$). At the same time, the Mg content was higher in the flowers than in fruits by 370.75% at 62.5 mg nCu/kg, by 282 % at 500 mg nCu/kg, by 229.74% at 62.5 mg bCu/kg, and by 224.58% at 500 mg bCu/kg ($p \leq 0.05$). Magnesium uptake and translocation is closely related to that of Ca in plants, anatomically and physiologically, and so the same principles apply. Magnesium concentration in alfalfa plant tissues was observed to gradually reduce over the growth stages (Marković et al., 2009). This aligns with our observation of Mg concentration. Once again, we do not attribute the finding to the Cu treatments. An important point is noted here that phloem and not the xylem is responsible for the transport of the nutrients (photosynthates) from leaves to the storage organs, like fruits (Wilkinson et al., 1987).

Sulphur

The S concentration in the flowers was higher than control by 77.59% at 62.5 mg nCu/kg and by 93.99% at 62.5 mg bCu/kg ($p \leq 0.05$) (Figure 3.6c). Additionally, the S content in the flowers was significantly higher than that in the bell pepper fruit by 221.64% at 62.5 mg nCu/kg, by 138.26% at 500 mg nCu/kg, by 173.33% at 62.5 mg bCu/kg, and by 168.56% at 500 mg bCu/kg ($p \leq 0.05$). Monaghan et al. (1999) exposed wheat plants to different sulphate isotopes, hydroponically, and figured the S concentration in the shoot tissue to decline over the period of growth. According to a review on sulphur in crop production, Scherer (2001) noted that the plants have high S sinks in the early stages of growth, which deplete with further plant development. Just like the explanantion under the Ca section, it is thought the rapidly growing fruit takes up the S storage for the biochemical and developmental processes.

Growth hormones like auxins, cytokinins, and gibberellins play an instrumental role especially at the reproductive stage. Copper plays an important part in converting tryptamine to an auxin, an indoleacetic acid (Schneider & Wightman, 1974, Salisbury & Ross, 1992). Overall, data suggests think that the Cu ammended to the soil supported the development of flower to fruit. Hence, there was no significant increase in fruit Cu concentration under the treatments. This leverages our hypothesis that nCu/Cu was beneficial for plant health and fruit production.

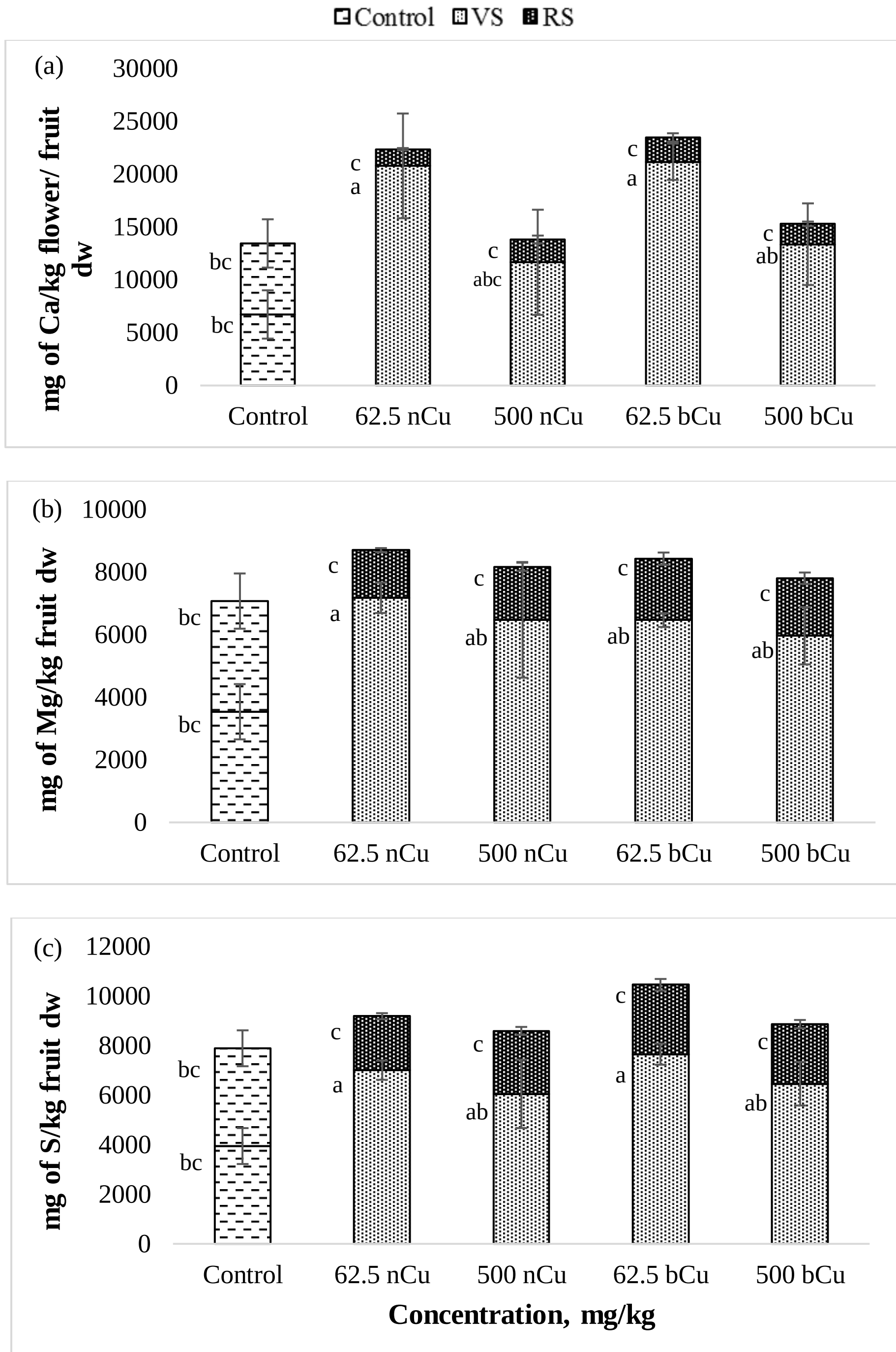


Figure 3.6: Comparison of the nutrients (a) Ca (b) Mg and (c) S in the fruit/ flower tissue in bell pepper plants cultivated under nCu and bCu treatments at 0, 62.5, and 500 mg of Cu/kg soil. Data are averages of three replicates ± SE. Averages without a common letter are significantly different with respect to each other, as per one-way ANOVA and Tukey test ($p \leq 0.05$)

3.3.5 Gas exchange

The gas exchange data (measured by evapotranspiration, stomatal conductance, and photosynthesis) for the VS had no influence of the Cu treatment and have been shown on Figure A3.1 (Annex II). The gas exchange data for RS are given in Figure 3.7. At 500 mg/kg, evapotranspiration (E) was 30.54% higher in nCu treated plants, compared to bCu ($p \leq 0.05$, Figure 3.7a). At the same concentration, stomatal conductance (g_s) for nCu treatments was 47.47% higher than control and 51.31% higher, compared to bCu treatments (Figure 3.7b, $p \leq 0.05$).

As seen in Figure 3.4a, at 500 mg/kg treatment, Cu was much better translocated to the leaf in nCu treatments, compared to bCu. Bearing in mind that gas exchange measurements were taken in fully mature plants, right before harvesting, this result correlates to the E and g_s findings at the same concentration. Additionally, the soil dissolution study (Figure 3.1) supports the finding since the rate of dissolution at 500 mg/kg was 153.12% higher for nCu, compared to bCu treatment after 48 h of dissolution ($p \leq 0.05$). The ionic form of nCu apparently easily traversed through the plant vascular system, translocated, and bio-accumulated in the leaf, influencing E and g_s. At the concentrations tested. This could be indicative of beneficial effects of nCu, since Cu plays an important role in photosynthetic electron transport (Yruela, 2005).

However, our findings are contrary to those obtained by Musante and White (2012), where they saw a reduction (compared to control) in hydroponically grown *Cucurbita pepo* transpiration, under nCu treatment. This could be due to differences in the growth matrix, plant species, and plant growth stage. Plant root exudates in a soil matrix tend to interact with the amended Cu particles, which facilitates the Cu ion transport (Zhao et al., 2016). In hydroponic studies, on the other hand, Cu tends to precipitate as phosphates, carbonates, and /or hydroxides (Musante & White, 2012).

Photosynthesis (P_n), at 62.5 mg bCu/kg, was observed to be lower than control by 27.98% (Figure 3.7c, $p \leq 0.05$). At such concentration, P_n was observed to be higher by 42.33% for nCu compared to bCu ($p \leq 0.05$). This result aligns with Hong et al. (2016)'s findings in soil grown cucumber plants and bCuO foliar application. Evidently, bCu had toxic influences on plant photosynthesis at low concentrations. This corresponds to the Cu translocation and leaf bioaccumulation trends shown in Figure 3.5a.

Looking at the gas exchange results overall, the nCu particles adsorbed on the plant root surfaces could be affecting the water transport, as observed by Asli and Neumann (2009) in TiO_2 treated corn plants. What we have observed are stimulatory effects of nCu in gas exchange, against inhibitory effects of metal/ENP treatments, previously reported (Zhao et al., 2013, Rawat et al., 2018).

3.3.6 Enzymatic activity and fruit nutrient quality (RS)

Catalase (CAT) and ascorbate peroxidase (APOX) activities in the root and leaf at plant maturity are given on Figure A3.2 (Annex II), where no significant effect of the treatments were observed. The fruit pigments at harvest (vitamin C, chlorophyll a, chlorophyll b, carotenoid/vitamin A) (Figure A3.3, Table A3.4, Annex II), and total sugar (Table A3.5, Annex II) were determined as parameters of fruit nutrient quality. None of them showed a significant effect of the treatment.

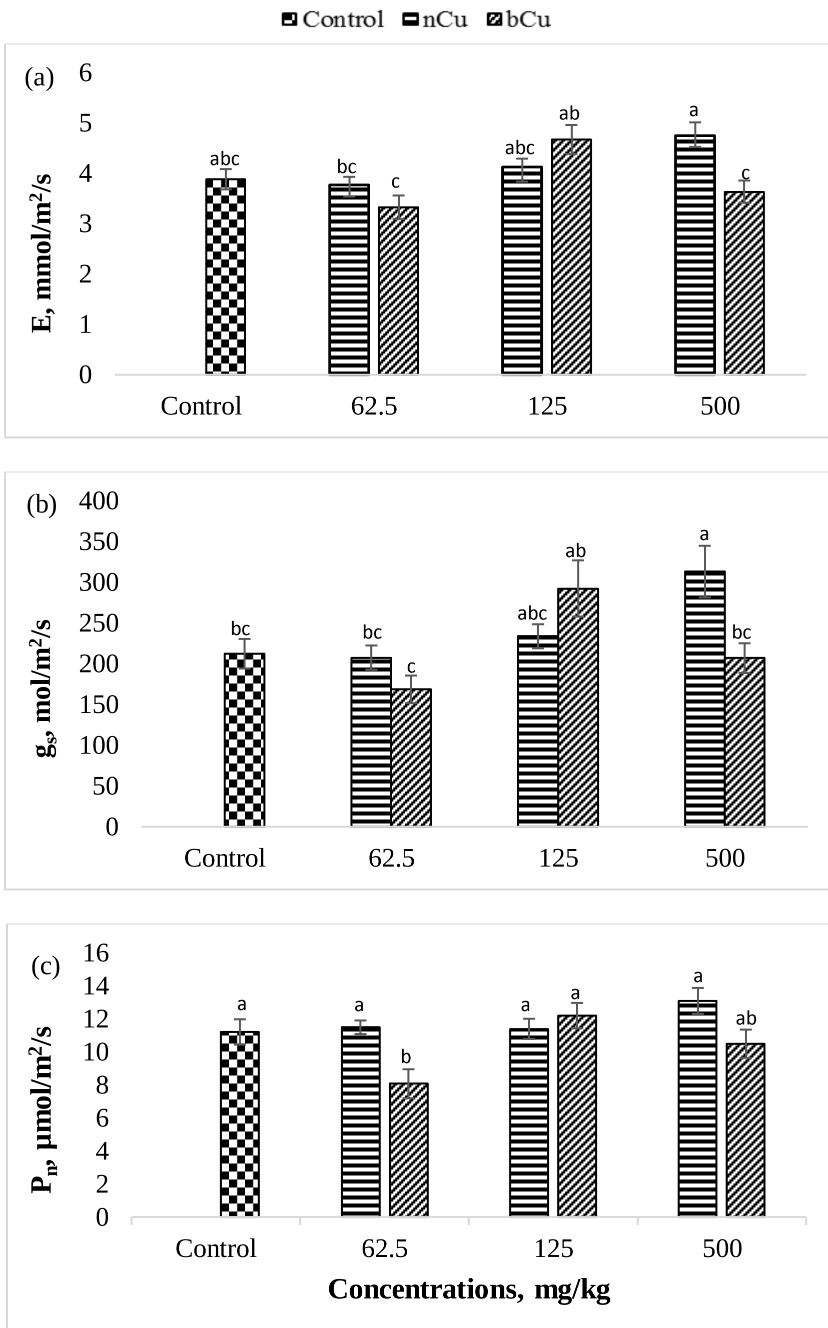


Figure 3.7: Gas exchange parameters (a) evapotranspiration, (b) stomatal conductance, and (c) photosynthesis for the reproductive stage study (RS) in bell pepper plants cultivated under nCu and bCu amended soil for 90 days at 0, 62.5, 125, and 500 mg of Cu/kg of soil. Data are averages of three replicates ± SE. Averages without a common letter are significantly different with respect to each other, as per one-way ANOVA, followed by Tukey test ($p \leq 0.05$)

3.4 Conclusions

Data from this study has shown that particle size of metallic Cu has a significant effect on plant growth and Cu bioaccumulation at VS and RS in bell pepper plants. The nCu (predominantly 500 mg/kg) treatment showed growth stimulatory effect on the plant root for the VS study. At the same time, Cu uptake and bioaccumulation were significantly higher in the plant tissue (especially leaf) from bCu treatment exposure. For the RS study, conversely, the Cu bioaccumulation was significantly higher at 500 mg nCu/kg treatment in the root and leaf tissue. The study indicates varying uptake tendencies of nCu and bCu at the two exposure periods in bell pepper plants. Gas exchange parameters, photosynthesis, stomatal conductance, and evapotranspiration were also significantly higher for the nCu treatments, compared to the bulk counterpart. The ascorbate peroxidase and catalase enzyme activities in bell pepper root and leaf for the full life-cycle exposure did not show any significance. As mentioned earlier, fruit nutrient quality in terms of carotenoid, chlorophyll, total sugar, and vitamin C content, at 90 day exposure, were not affected by the Cu treatments. Some of the findings indicate that at optimal concentrations and short term exposures (45 days), nano-sized copper has the potential to be used as a plant growth promoter. However, the conclusion needs to be corroborated by further experimental work.

Chapter 4: Soil weathered CuO nanoparticles hamper foliar health and pigments in spinach (*Spinacia oleracea*) plants

4.1 Introduction

Soils and sediments form an important environmental sink, besides majorly supporting agricultural practices. Copper based engineered nanoparticles (ENPs), thus, find their way into soil through intentional use (agricultural amendments) as well as incidental exposure (household waste disposal) (Adisa et al., 2018, Pullagurala et al., 2018). Being part of the soil biota, terrestrial plants run the risk of being affected by the exposure (Reddy et al., 2016, Ochao et al., 2018). The risk of adverse effects depends on various factors, like the plant species, time of exposure, concentration of the ENPs, composition of the matrix, and presence of co-contaminants, among others (Cota-Ruiz et al., 2018). Myriad of literature elaborates plant and ENP interaction and the resultant effects (Zuverza-Mena et al., 2017, Du et al., 2017). Variation in the exposure conditions could be affecting these interactions and effects. One such condition is the weathering/aging of ENPs in soil (Servin & White, 2016). ENPs are suspected to undergo various surface chemistry transformations in soil, like ionization, oxidation, sulfidation, aggregation, ROS formation, interactions with organic matter, and other chemical species (Rawat et al., 2018, Dimkpa et al., 2018). Along with it comes the possibility of altered effects on the plant species in terms of physiology, biochemistry, essential nutrients, and genetic content, compared to that of freshly applied ENPs.

Two important studies have come up in the recent past demonstrating the effect of aging of ENPs on soil grown crop plants. Servin et al. (2017) conducted an experiment on the effects of weathered vs unweathered CuO compounds on lettuce plants and their trophic transfer tendencies. This led to a 214% higher root Cu concentration in aged vs unaged nCuO treatments. Both ionic and bCuO treatments had opposite trends from nCuO. µ-XRF analysis showed that the CuO in

aged nano treatments turned into Cu-S and oxide complexes in the root tissue, whereas the bulk treatments had stayed as CuO. Another study analyzed the effects of aged vs unaged ZnO NPs and ionic Zn on wheat plant in terms of the grain productivity and metal accumulation (Dimpka et al., 2018). The fresh compounds tended to give a better bioaccumulation (root-to-shoot) and higher Zn accumulation in the grains as compared to the aged ones. Besides this, significant amount of work has been done on the effects of copper based ENPs with various plant species at different growth stages (Hong et al., 2015, Zuverza-Mena et al., 2015, Zhao et al., 2016, Apodaca et al., 2017, Ochao et al., 2017, Rawat et al., 2018, Du et al., 2018, Tamez et al., 2019).

Copper has been used for its antimicrobial properties since time immemorial. It was first used in a pesticide formulation, Bordeaux mixture in 1885 (Lamichhane et al., 2018). The increasing use of nano-form copper compounds holds further promises for the future of agriculture and its peripheral branches (Apodaca et al., 2018, Rawat & Peralta, 2018). Along with the benefits, plant and animal biota run the risk of direct exposure to Cu species, their bioaccumulation and bio-magnification in the food chain through overexposed food crops (Bonilla-Bird et al., 2018). Spinach (*Spinacia oleracea)* has been selected as the model plant for the study, since it ranked second in the list of dirty dozen vegetables and fruits (EWG, 2017; https://www.ewg.org/release/2017-dirty-dozen-strawberries-spinach-top-ewgs-list-pesticides-produce#.W70sZvlMFhE, last accessed: October 11, 2018). This implies that spinach carries significant amount of external pesticide residue along with the uptaken contaminants. This may pose a health risk to the consumers. Additionally, spinach is an important vegetable widely consumed across the globe. It is an important source of iron and provitamin A carotenoids in the human diet (Tang, G., 2010). The world production of spinach has been rising at an average rate

of 6% annually since 2006, with nearly 26 million tons produced in 2016 (http://www.fao.org/faostat/en/#data/QC, last accessed: October 12, 2018).

The possible effects of soil weathered ENPs on plants is a relatively new concept. By means of this study, we are attempting to add to the existing body of literature on food safety and/or beneficial effects of ENPs, specifically on copper. The findings from studies like these may support regulations on the manufacture and use of yet unregulated copper based ENPs in agriculture. The study is also an attempt to contribute to ENP studies databases that help generate *in-silico* predictive tools.

4.2 Method and materials

4.2.1 Characterization of the copper-based chemicals

Nano-particulate copper oxide (nCuO) and bulk copper oxide (bCuO) were procured from Sigma Aldrich (St. Louis, MO). The characterization of the chemicals is as given on Table A4.1, Annex III (Hong et al., 2015, Lin et al., 2015). $CuSO_4$ used for ionic treatment and was purchased from Spectrum (New Brunswick, NJ). All compound were used without further purification.

4.2.2 Growth matrix and plant culture at the green house

Plant culture was conducted at Texas A&M Agrilife Research Centre greenhouse (El Paso, TX) under controlled environmental conditions. The spinach seeds, Seaside F1 (Johny Selected Seeds, Winslow, ME) were tested for germination on petri dishes and the viability was estimated to be 67%. They were grown in starting mix and were transplanted as five week old seedlings into pots with amended soil, two kg in each pot, two seedlings per pot. The soil used was a mix of natural soil and potting mix (2:1). Natural soil was collected at a cotton growing field in Socorro, TX (latitude: N 31°41' 30.705” and longitude: W 106°17' 13.936”, elevation: 1,115 m asl) from upto 0 to 30 cm soil depth. It was characterized to be silty loam (65% silt, 20% sand, and 15% clay) (Ryżak et al., 2011, Rawat et al., 2018) and was fortified with organic matter for improved

aeration. The treatments used were 0 (control), 400 400, or 40 mg/kg of nCuO, bCuO, or $CuSO_4$, respectively. A set of three replicates each of these treatments were set up for weathering for five weeks at the green house, the soil being kept at field capacity. Pots were prepared with the soil mix by manually homogenizing with measured amount of chemicals in a DI water suspension. Pots identical to the ones that underwent weathering were prepared as fresh/unaged/unweathered treatments at the end of five weeks, when spinach seedlings were transplanted. The plants were regularly watered with 100 ml DI water/kg of soil mix and occasionally treated with Avid 0.15 (Active ingredient, abamectin 2% v/v) for possible pests and insects. The average relative humidity at the green house during the 45 days' plant growth was 23.83 ± 0.18%. The average AM temperature was recorded to be 23.17 ± 0.12 ˚C and the average PM temperature was 26.75 ± 0.1125 ˚C. Additionally, the average daylight was recorded as 91.97 ± 2.11 mol m^{-2} d^{-1}. Gas exchange measurements, enzyme and pigment extracts were prepared at regular growth intervals of the plants.

4.2.3 Gas exchange measurements

Net photosynthetic rate, stomatal conductance, transpiration rate, intercellular CO_2 concentration, water use efficiency, and vapor pressure deficit were considered as measures of gas exchange. Data were collected at various intervals during plant growth (10, 20, and 40 days post transplantation) with a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury, Massachusetts, USA). The instrument probe contains a leaf cuvette used to hold a new fully expanded leaf from each plant and take the measurement. The plants were irrigated before the measurements were taken between 1000 to 1400 h on sunny days. Any cloud cover interferes with normal respiration and photosynthesis of the plants (Zhao et al., 2013).

4.2.4 Enzyme analysis

Leaf and root fresh tissue extracts were prepared in 25 mM, for catalase (CAT), and 0.1 M phosphate (KH_2PO_4) buffer (pH: 7.4) for ascorbate peroxidase (APOX) analysis. These extracts were run to measure enzyme kinetic activity by UV-visible spectrophotometry at 240 nm (Murgia et al., 2004, Gallego et al., 1996).

4.2.5 Pigment content (carotenoid, Chl a and b) analysis

Spinach leaf extracts were prepared in 80% acetone and leaf pigments were estimated by measuring absorbance on a plate reader. The method used was as suggested by Lichtenthaler and Wellburn (1983). The plate reader used was SPECTRAmax190 by Molecular Devices (Sunnyvale, CA).

4.2.6 Harvest and elemental analysis

The spinach plants were grown for 45 days post transplantation under the treatments. The total foliar area, total number of leaves, and their total weights were recorded for each plant. The total foliar area was measured by LI-3100C area meter (LI-COR Biosciences, Lincoln, Nebraska, USA). The separated samples were washed in 0.01M HNO_3 followed by DI water. The root tissues were also collected separately, cleaned, weighed, measured, and dried. After air drying, the fresh samples were oven dried at 60-70˚C for 72 h (Rawat et al., 2018).

The dried plant samples were weighed to determine the dry biomass. Samples were ground and the powders were digested in a digiPrep hot block (SCP Science, Quebec, Canada) for further analysis. The Environmental Protection Agency (EPA) method 3051 was employed for digestion using plasma pure nitric acid (65%) and hydrogen peroxide (30%) (1:4). Inductively coupled plasma-optical emission spectroscopy (ICP-OES, Perkin-Elmer Optima 4300 DV, Shelton, CT) was used to determine the macro and micro elements (Ca, K, Mg, P, S, Fe, Zn, Cu, Mn, Ni, Mo) concentrations in the digested samples (Zuverza-Mena et al., 2015). Three blank sample carriers,

two carriers with spinach as the standard reference material (NIST 1570a, Gaithersburg, MD) and three samples spiked with 20 mg/L of the Cu NPs were all digested and included in the analytical process as part of the QA/QC (Hong et al., 2015, Rawat et al., 2018).

Standard reference material 1570a from the National Institute of Standards and Technology was used to evaluate the extraction efficiency of the system, obtaining a recovery of 101.11 ± 5.59%. For quality control of the ICP readings, blank and a spiked sample containing Cu 20 mg/L were read every 25 samples. The average readings for Cu in the spiked sample was 21.30 ± 0.58 mg/L, thus validating the digestion process. The ICP-OES parameters used were as follows: nebulizer flow, 0.80 L/min; power, 1,400 W; peristaltic pump rate, 1.5 mL/min; flush time, 20 s; delay time, 20 s; read time, 10 s; and wash time, 60 s.

4.2.7 Statistical analysis

All the data collected were analyzed using SPSS (IMB) program. The mean values from the controls and the treatments were compared among each other using one-way ANOVA followed by Tukey-Kramer multiple comparison test (SPSS 19.0 package, Chicago, IL). Results are presented as mean ± standard error and were observed for statistically significant differences at $p \leq 0.05$.

4.3 Results and discussion

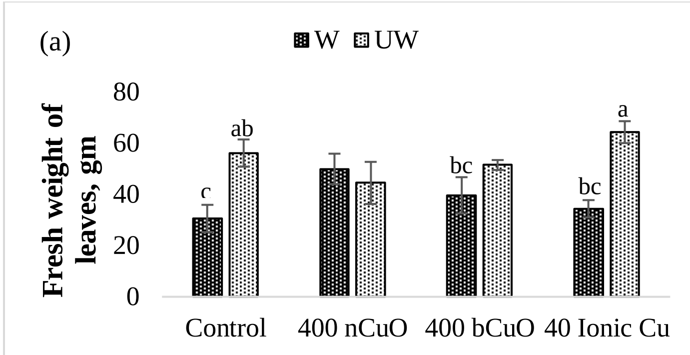
4.3.1 Physiological effects (agronomy, copper uptake and translocation)

The physiological effects of W and UW copper treatments on spinach plants are given in this section. Foliar health of the plant at 45 days (Figure 4.1), Cu bioaccumulation in the leaves (Figure 4.2), and significant differences in gas exchange (Figure 4.3) have been explained.

As shown on Figure 4.1, fresh foliar biomass in spinach plants for the unweathered treatments was higher in control by 83.55% and in ionic treatment by 87%, compared to the corresponding weathered treatment. This effect of aging was visible at 45 days of plant growth for

control (Figure A4.6, Annex III) and for ionic Cu treatments (Figure A4.7, Annex III). In addition, the foliar area in UW ionic treatment was higher than the corresponding W treatments by 77%; however, in both cases the differences were significant only at $p \leq 0.1$. It is possible that increasing the number of repetitions will give statistical significance at a lower probability. A possible rationale behind the detrimental effect of weathering is that it probably limited the aeration in the soil and subdued the plant nutrients.

Soil organic matter (OM) in most agricultural soils is around 3-6% (http://franklin.cce.cornell.edu/resources/soil-organic-matter-fact-sheet, last accessed: October 16, 2018). The OM content in the soil used in this study was close to 8% and pH 7.0. The soil used is likely to have a high CEC (>15 meq/100 g), given the high OM content (https://extension2.missouri.edu/MG4, last accessed: October 16, 2018). It was supposed that most of the nCuO added aggregated with the OM, or was taken up and bio-accumulated in the shoot post dissolution (Figure 4.2), both for W and UW categories (Gao et al., 2017). Hence the agronomical parameters and Cu concentration were not affected by W/UW CuO treatments. An important point to be noted here is that the plant foliar health was not affected by nano/bulk treatment and the process of weathering, which speaks of its utility as a sturdy agricultural amendment for antimicrobial use in spinach cultivation.



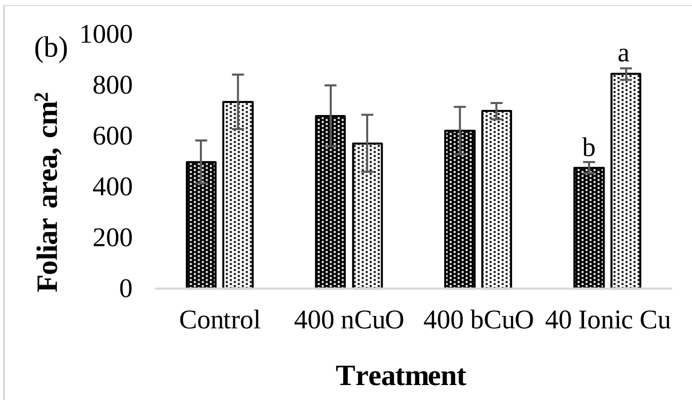


Figure 4.1: Fresh foliar biomass (a) and foliar area (b) of spinach (*Spinacia oleracea)* plants grown in weathered vs unweathered control, nCuO, bCuO, and ionic Cu at 0, 400, 400, and 40 mg/kg treatments, respectively in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of three replicates ± SE. Average values with uncommon letters are statistically significantly different compared to each other, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test ($p \leq 0.1$).

Weathering did not have an effect on the copper uptake and bioaccumulation in spinach leaves across the treatments. Despite that, not considering weathering, effect of the Cu treatments was evident in leaf Cu bioaccumulation (Figure 4.2). At 400 mg/kg nCuO, the shoot Cu concentration was significantly higher than control by 275 % under W treatment and 270 % under UW treatment ($p \leq 0.05$). Similarly, at 400 mg/kg bCuO treatment, the shoot Cu concentration was significantly higher than the control by 232% for the W treatment and 141 % for the UW treatment

($p \leq 0.05$). It is worth mentioning that the spinach root Cu concentration did not show any significant difference among the various treatments (including W/UW) (Figure A4.1, Annex III). Servin et al. (2017) had contrary findings with lettuce plants growing in similar treatments. Root Cu content had clear effects of weathering with significantly lower Cu concentration in W (nano and bulk) CuO treatments, compared to UW counterparts. The opposite findings could be attributed to the differences in soil type, plant species, duration of weathering (5 weeks *vs* 70 days), and the growth conditions. Copper bioaccumulation in spinach plant (barring the effect of weathering) in the current study align with findings in cilantro experiment by Zuverza-Mena et al. (2015). They observed that Cu from the soil treatments was translocated to the shoot with significant differences in the leaf Cu bioaccumulation among the treatments. However, no significant differences were found in the root Cu content.

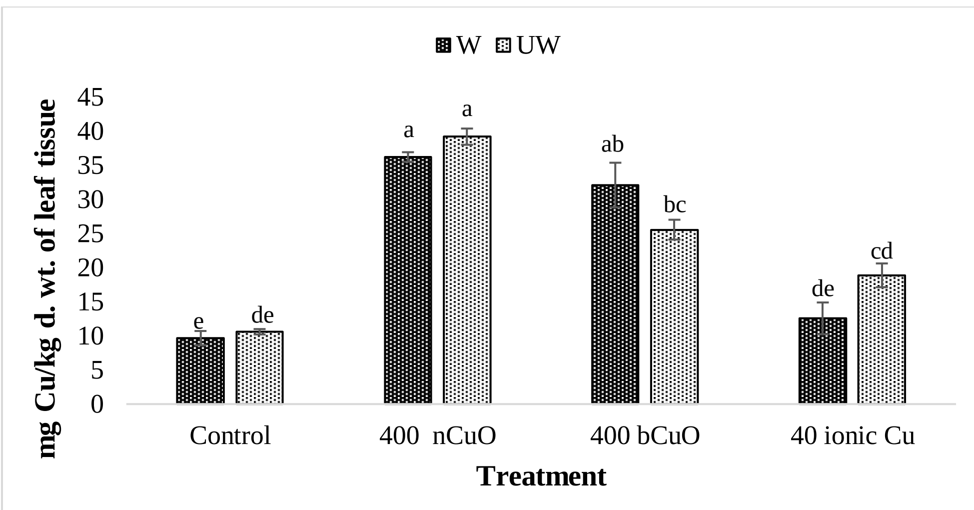


Figure 4.2: Copper bioaccumulation in spinach (*Spinacia oleracea)* leaves of plants grown in weathered vs unweathered control, nCuO, bCuO, and ionic Cu at 0, 400, 400, and 40 mg/kg treatments, respectively, in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of three replicates ± SE. Average values with uncommon letters are statistically significantly different compared to each other and the control, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test ($p \leq 0.05$)

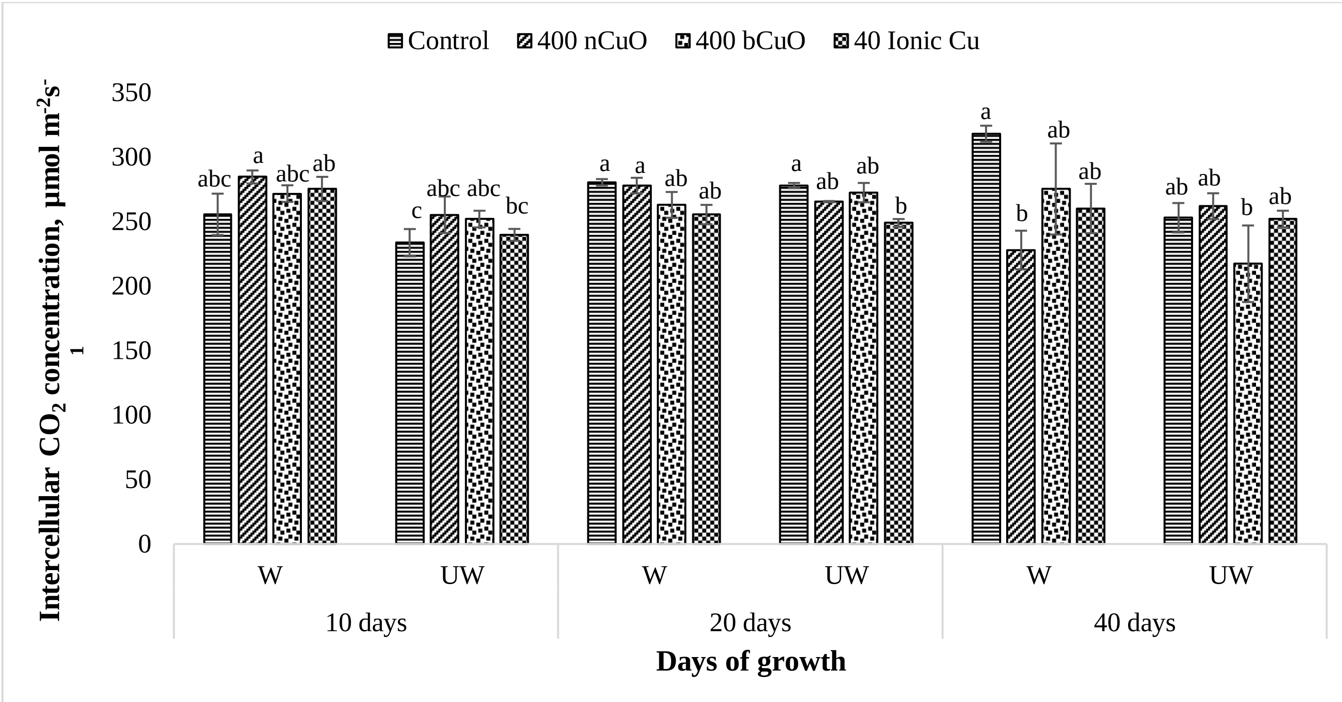


Figure 4.3: Intercellular CO_2 concentration at various time points during the growth cycle of spinach (*Spinacia oleracea)* plants grown in weathered vs unweathered control, nCuO, bCuO, and ionic Cu treatments at 0, 400, 400, and 40 mg/kg, respectively, in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of three biological replicates ± SE. Average values with uncommon letters are statistically significantly different with respect to each other within each exposure period, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test ($p \leq 0.1$).

4.3.2 Gas exchange

Various gas exchange parameters analyzed include stomatal conductance, photosynthesis, transpiration rate, water use efficiency, vapor pressure deficit, and intercellular CO_2 concentration at three different exposure periods of 10, 20, and 40 days. None of the measured values showed significant differences among each other, or with the control at 10 days. However, at 20 days, for the UW treatments, ionic Cu had a 10.43% lower intercellular CO_2 concentration (C_i), compared to control; this value was statistically different at $p \leq 0.1$. This testifies the toxic effect of ionic Cu, most easily ionizable and bioavailable of the Cu species used. Our previous work with bell pepper had similar reduction in gas exchange parameters in ionic Cu treatment (Rawat et al., 2018). Other studies have also discussed about the bioavailability and toxic effect of ionic Cu in plants (Eaton, 1942, Julich & Gath, 2014). At the 40 days exposure period, Ci was lower in W nCuO treatments by 28.35%, compared to W control. As in the case of 20 day exposure, the difference was statistically significant at $p \leq 0.1$. This broadly indicates a toxic effect of the nano Cu treatment in the spinach plant, where bulk was not really affecting the gas exchange or photosynthesis in the leaves. Of the two CuO species used in the experiment, nCuO goes more easily into solution, yielding a much higher Cu ion concentration in the soil solution, according to the soil dissolution study given in our previous work (Rawat et al., 2018). Ouzounidou et al. (1998) exposed spinach roots to 160 µM Cu (ionic source) for seven days and observed very similar reduction in C_i, compared to control. Copper from the W nCuO treatment was apparently more available to the plant over the long term exposure (effect of both weathering and 40 days growth in treatment) but showed no effects in curtailing the C_i over the short term (10/20 days or UW counterparts). This is also consistent with Gao et al. (2017), who mention that Cu from nCuO becomes bioavailable for plant uptake gradually over time, unlike the ionic treatment that is readily bioavailable. No effect was observed at the 10 day exposure period. Vinit-Dunand et al. (2002) cultured cucumber

plants in 10 µg/g Cu treatment (ionic source) for five days but noticed no significant effect on the intercellular CO_2 mole fraction compared to control.

Additionally, significant effects of the Cu treatments were observed for the transpiration rate in spinach leaves at 20 days. As given on Figure A4.5 (Annex III), W bCuO had a higher transpiration rate by 36.8% compared to the W ionic treatment ($p \leq 0.1$). Ionic Cu treatment apparently reduced the transpiration rate being toxic for the same reasons mentioned above. Additionally, bCuO took some time to go into solution, being the UW category and the slowest to go into solution among the three Cu species (Rawat et al., 2018). No significant differences were observed in the spinach leaf transpiration rate at other exposure periods of 10 and 40 days.

4.3.3 Biochemical effects

Biochemical effects have been quantified in terms of the root enzyme activity at plant harvest, leaf enzyme activity at various time points over the plant life cycle, and leaf pigment content across the growth period. Catalase and ascorbate peroxidase have been indicated to be scavenging enzymes acting on reactive oxygen species like H_2O_2 in plant cellular systems responding to stress, converting it to water and oxygen (Mukherjee et al., 2014). The two pigments elaborated carotenoid (vitamin A) and chlorophyll a are related to each other and help quantify the leaf health and nutrition (Lichtenthaler & Wellburn, 1983).

Enzymes

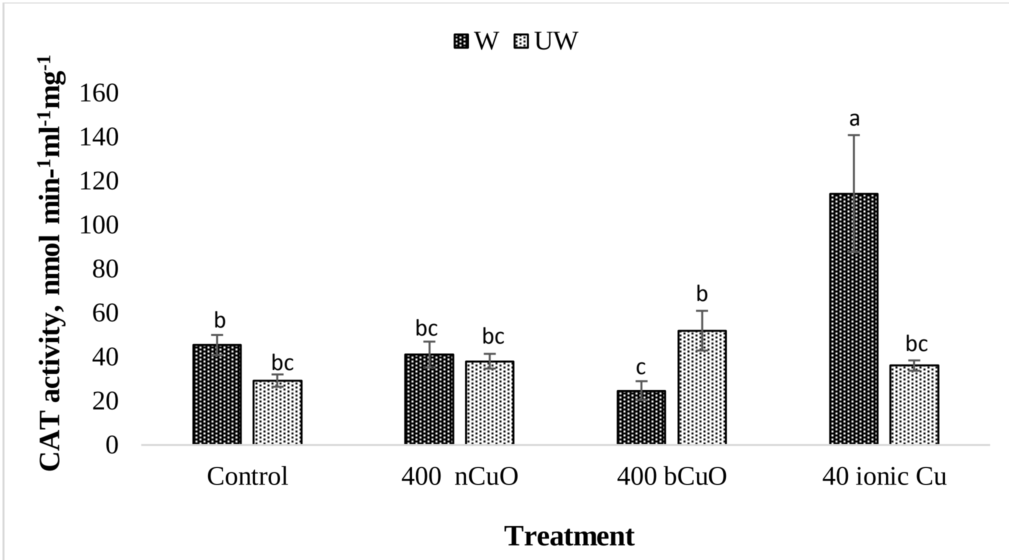


Figure 4.4: Catalase activity in spinach (*Spinacia oleracea)* roots measured at 45 days post transplantation. The plants were grown in weathered vs unweathered control, nCuO, bCuO, and ionic Cu treatments at 0, 400, 400, and 40 mg/kg, respectively, in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of three replicates ± SE. Average values with uncommon letters are statistically significantly different with respect to each other, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test ($p \leq 0.05$).

Root catalase activity, measured at 45 days of plant growth (cellular photosynthates saturation), was higher for UW bCuO treatment by 110 %, compared to the corresponding weathered one. Conversely, it was higher for the weathered ionic treatment by 216%, compared to the unweathered one ($p \leq 0.05$). Additionally, under the weathered conditions, the root catalase for bCuO treatment was under produced by 84% and that for the ionic treatment was over produced by ~151.26%, compared to control ($p \leq 0.05$). The ionic treatment was apparently most potent in creating stress and toxicity in the spinach root, under the W category. A comparison of the results for bCuO and ionic Cu treatments on Figures 4.2 and 4.4, showed that they were relatable. Copper from W ionic Cu treatment was taken up lesser in the spinach shoot, compared to its UW

counterpart (though not statistically significant) (Figure 4.2). Hence, it resulted in a significantly higher catalase activity in the root in W ionic treatment, opposite results being seen in Figure 4.4. Similarly, Cu from W bCuO treatment was better translocated to shoots than from its UW counterpart (though not statistically significant) (Figure 4.2). Opposite effect was observed in the bCuO catalase activity in the roots. A similar observation can be made about the bCuO treatment, compared with control, under the W category. Catalase activity in W bCuO treatment was similar to the observation made by Ochao et al. (2017) in roots of pea plants tested after 45 days of exposure to 50 and 100 mg/kg bCuO.

Additionally, nCuO treatments did not affect the root catalase activity. It is assumed that most of the nCuO aggregated with soil OM (~8%) and/or taken up and translocated to the aerial parts of the plant (Figure 4.2). Table A4.3 (Annex III) shows the Cu concentration in soil samples after the plants were harvested. Copper in nCuO amended soil samples was significantly higher by 39.69% as compared to soil samples from bCuO treatments ($p \leq 0.05$). This corroborates higher aggregation of nCuO compared to bulk and hence no effects on root enzyme activity were observed.

In general, a gradual increase in the leaf APOX activities over the growth cycle of the plant was observed (Figure 4.5). The APOX activity at 10 days post-transplantation in the spinach leaf was found to be 219 % higher in UW ionic treatment, compared to the W ionic treatment ($p \leq 0.05$). Of that, the UW ionic treatment had significantly higher enzyme activity since it got little time in soil to aggregate, unlike the W one. Ionic Cu was apparently most actively absorbed by the plant since it was in ionic form; thus, increased enzyme activity (Eaton, 1942). Cu ions from the CuO treatments are likely to go gradually into solution (Rawat et al., 2018). Tamez et al. (2019),

similarly, saw increased APOX activity in sugarcane leaves from a yearlong ionic Cu exposure at 20 and 60 mg/kg.

Referring to Figure A4.4 in the Annex III, the leaf catalase data showed a trend of gradually increasing enzyme activity over time, similar to leaf APOX (Figure 4.5). The increase was not statistically significantly although. Likewise, under each exposure period, leaf catalase activity data from controls was higher than the Cu treatments. Trujillo-Reyes et al. (2014) tested catalase and APOX activities in hydroponically grown lettuce plants and observed a significantly lower activity than control at high nCu concentration in the leaf. This aligns with leaf catalase (Figure A4.4, Annex III) and APOX (Figure 4.5) findings for each individual exposure period, though not statistically significant. Zhao et al. (2012) found opposite trends in catalase and APOX enzyme activity in corn seedling leaf in $nCeO_2$ treatment with decreasing trends over the exposure intervals and an overall drop in activity over the growth/study period.

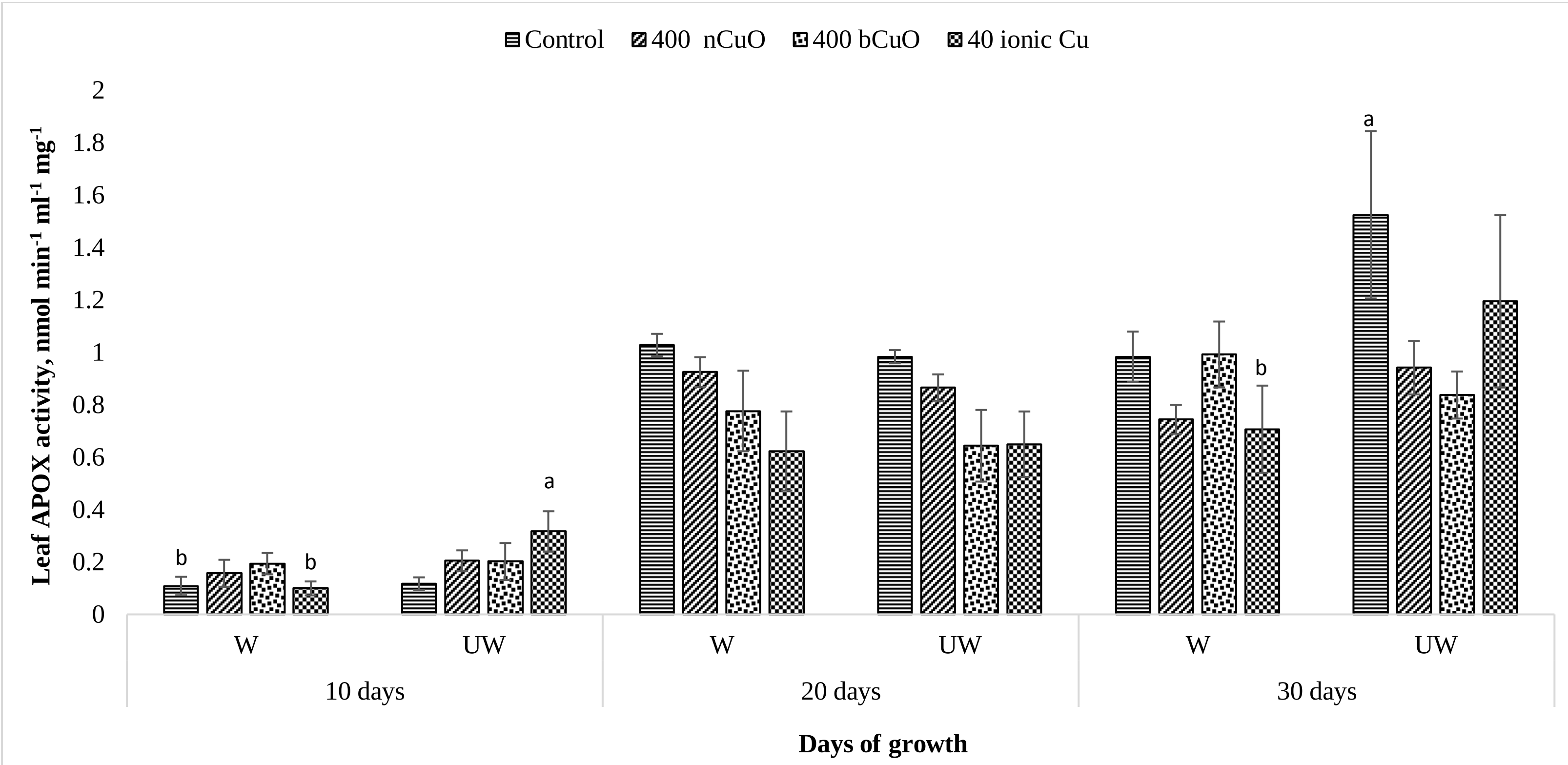


Figure 4.5: Leaf APOX activity at various time points during the growth cycle of spinach plants grown in weathered vs unweathered control (0), 400, 400, and 40 mg/kg nCuO, bCuO and ionic Cu treatments in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of three biological replicates and three subsequent technical replicates ± SE. Average values with uncommon letters are statistically significantly different with respect to each other within each time interval, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test ($p \leq 0.05$).

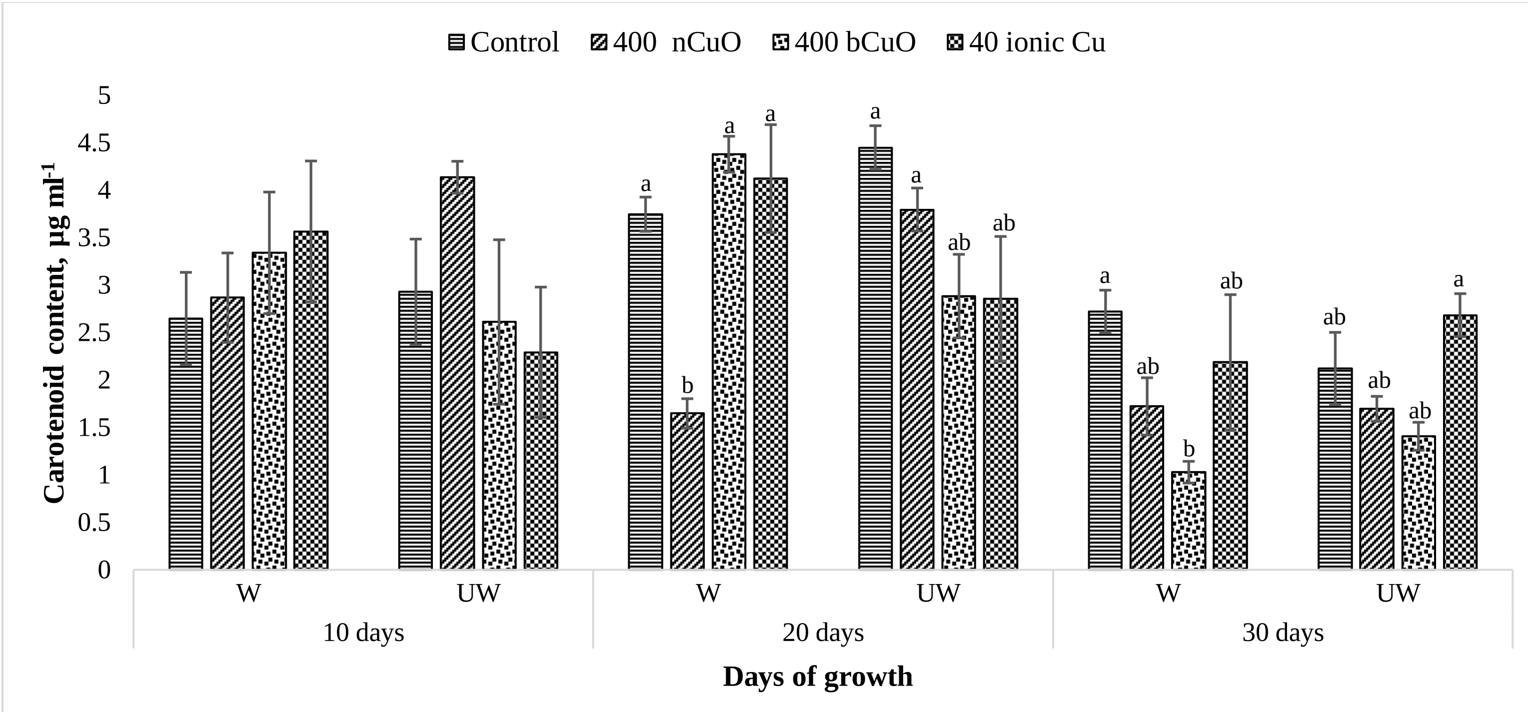


Figure 4.6: Leaf carotenoid content at various time points during the growth cycle of spinach plants grown in weathered vs unweathered control, nCuO, bCuO, and ionic Cu treatments at 0, 400, 400, and 40 mg/kg, respectively, in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of three biological replicates and three subsequent technical replicates ± SE. Average values with uncommon letters are statistically significantly different with respect to each other within each time interval, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test ($p \leq 0.05$).

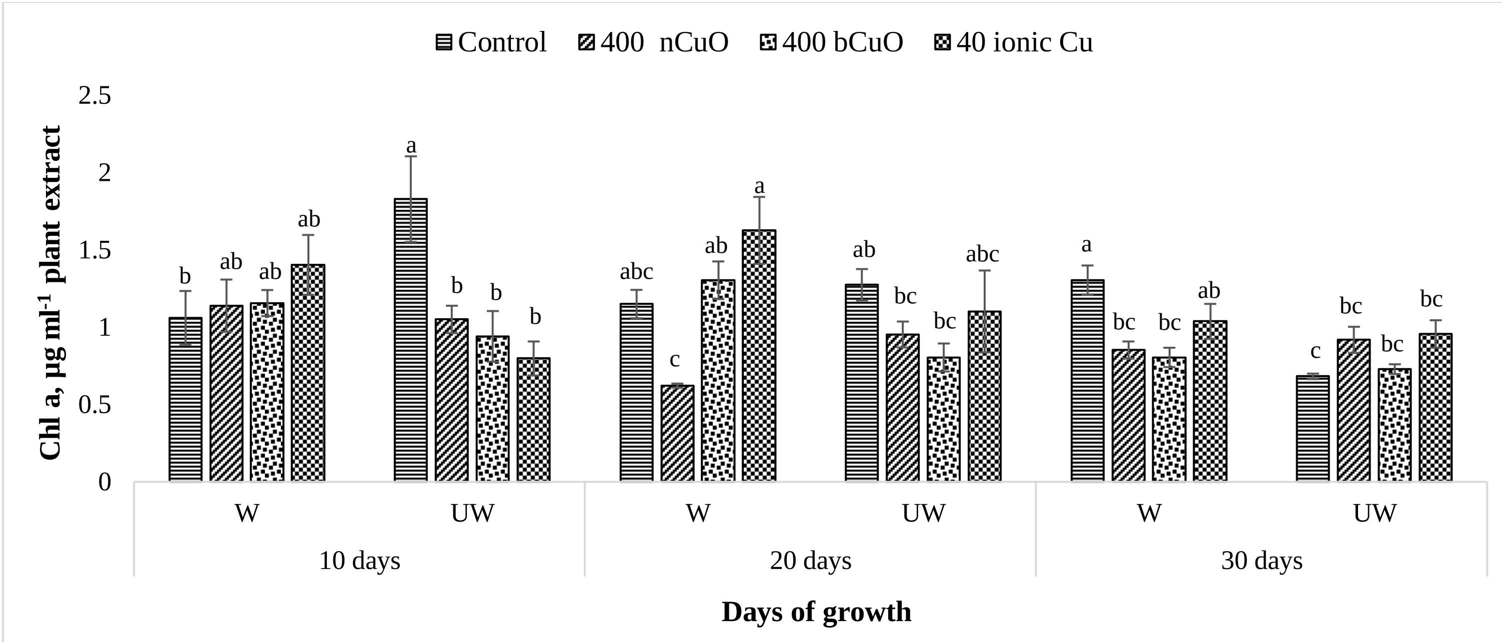


Figure 4.7: Comparison of the leaf Chl a content at various time points during the growth cycle of spinach plants grown in weathered vs unweathered control, nCuO, bCuO, and ionic Cu treatments at 0, 400, 400, and 40 mg/kg, respectively, in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of three biological replicates and three subsequent technical replicates ± SE. Average values with uncommon letters are statistically significantly different with respect to each other within each time interval, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test ($p \leq 0.05$)

Pigments and carotenoid (Vitamin A)

Leaf carotenoids were not affected by weathering or by any of the treatments at the 10 day exposure period (Figure 4.6). However, at the 20 day exposure period, the W nCuO treatment reduced them by 56%, compared with W control ($p \leq 0.05$). They were also lower than the UW counterpart by 57% ($p \leq 0.05$). At the 30 day exposure period, again, no effects of weathering were observed. Carotenoid content in the W bCuO treatment was, however, lower than W control by 62.23 % ($p \leq 0.05$). Thus, W nCuO apparently had an influence on the spinach leaf health at the middle of the life cycle. But the effect was countered by the plant's internal defense mechanism (Zhao et al., 2012). Additionally, under the W category, nCuO affected carotenoids at 20 days but bCuO was effective at the 30 day exposure period. Of the two Cu species used, nCuO goes more easily into solution, as compared to bCuO, giving a higher Cu ion concentration much sooner. This tendency resulted in nCuO and bCuO causing toxicity in the spinach leaf at an early (at 20 days for nCuO) and a later (at 30 days for bCuO) time point, accordingly. This has been verified in a soil dissolution study given in our previous work, where similar soil was used (Rawat et al., 2018). Also, CuO tends to go gradually into solution (post weathering) to become bioavailable over 20-30 days of plant exposure (Julich & Gath, 2014; McShane et al., 2014; Gao et al., 2017).

The literature describes Cu as an essential micronutrient in plants with defined roles in photosynthetic electron transport and mitochondrial respiration (Yruela, 2005). Botanists have considered Cu to be part of photosynthesis systems (PS) I and II. However, excess of Cu influences photosynthesis by affecting lipid and pigment biosynthesis (Baron et al., 1995). Chl *a* was differently affected at different treatments. At the 10 day interval, Chl a, under the UW control was 72 % higher than W control ($p \leq 0.05$). Additionally, UW control gave 43 %, 94 %, and 129 % higher Chl a, compared to UW nCuO, bCuO, and ionic Cu treatments, respectively ($p \leq 0.05$). A clear effect of weathering amongst the controls and the effect of the Cu treatments in the UW

category was observed. Saison et al. (2010) tested the effects of hydroponically exposed core-shell CuO NPs on green algae (*Chlamydomonas reinhardtii)* and observed a chlorophyll degradation by PS II photoinhibition. In addition, Prasad et al. (2001) analyzed the effect of Cu (2-50 µM ionic source) on *Lemna triscula* (duckweed) in a hydroponic environment, they saw a deterioration in Chl *a* and carotenoids, resulting in an inhibition of photosynthesis and gas exchange.

At the 20 day exposure period, under the W category, Chl *a,* under ionic treatment, was higher than nCuO treatment by 161 % ($p \leq 0.05$). Under the 30 day exposure period, the effect of weathering on controls was reversed, compared to 10 day exposure period. Chlorophyll a (Chl *a*), under the W control, was higher than that under UW control by 91 % ($p \leq 0.05$). Additionally, under the W category, Chl *a* in nCuO treatment was 53 % lower and that in bCuO treatment, and was 62 % lower than the control ($p \leq 0.05$). Hence, at the 30 day exposure period, both nano and bulk CuO significantly reduced the leaf chlorophyll and were toxic to the plant. This indicates a gradual release of Cu ions from the rhizosphere, negatively affecting the plant photosynthetic electron transport (Yruela, 2005). Post weathering, the CuO could have either aggregated with the soil OM, or been adsorbed on the root that lead to stabilization and slow ion release over time (Asli & Neumann, 2009, Sekine et al., 2017, Gao et al., 2017). Additionally, both the parameters carotenoid content (Figure 4.6) and Chl a (Figure 4.7), showed a significant reduction under W bCuO treatment, compared to W control at 30 day exposure interval. The basic observation about a reduction in the overall chlorophyll content lines up with the findings given by Ouzounidou et al. (1998), where they saw chlorophyll reduction in spinach leaves under 160 µM Cu exposure (ionic source). Additionally, Connan & Stengel (2011) made an observation with brown algae (*Ascophyllum nodosum* and *Fucus vesiculosus)* cultured hydroponically, where Cu exposure at 5 mgL^{-1} (ionic source) significantly inhibited photosynthesis.

4.4 Conclusions

Data from this study has shown that the effect of weathering of the (nano and bulk) CuO treatments are observable only in the aerial parts of the spinach plant and minimally in the root, except for bCuO showing significant root catalase activity. In addition, weathering did not have an effect on Cu bioaccumulation in the plant tissues. The shoot Cu concentrations reflected an effect of the Cu treatments but not of weathering.

However, the effect of weathering was visible in leaf health and pigments. Weathered control and W ionic treatments had significant ill effect on the foliar health of the plant. Weathered nCuO reduced carotenoid at 20 day exposure period, compared to both W control and the UW counterpart. This effect was not visible at 30 days exposure period. However, W bCuO gave significantly lower carotenoid in leaves compared to W control at the 30 day exposure period. Chlorophyll a (Chl a) was hampered by all the UW Cu treatments, compared to UW control at 10 days of exposure. The effect of weathering was evident between the controls at 10 day and 30 day intervals of exposure. The W CuO treatments gave significantly lower Chl *a* than W control at 30 days of exposure. The outcomes of both the pigments suggested that W CuO treatments exercised an influence on the plant foliar health at the mid and towards the end of the plant life cycle.

Overall, a variable influence of weathered Cu compounds on spinach plants grown in organic soil in a controlled greenhouse environment was observed. There was a clear effect of weathered CuO treatments on leaf pigments towards the end of the plant life cycle, suggesting a deterioration of the leaf quality. This suggests that relatively higher CuO concentration released to the environment could affect leaf quality of important edible plants. The study could be expanded to other plant species to corroborate the findings with Cu based nanoparticles.

Chapter 5: Conclusions

Taking a broad overview of the three experiments under the project, particle size of the Cu compounds had a significant influence on the Cu bioaccumulation in plant tissues and some other physiological effects. For the first two experiments with bell pepper plants, the amended copper majorly bioaccumulated in the root. Nano sized CuO inhibited the uptake of elemental nutrients, while bulk CuO stimulated the translocation of most of the nutrients to the aerial parts of the bell pepper plant including fruit. This finding was in keeping with the initial hypothesis that nano sized Cu has toxic effect on the plants. The second experiment about the interaction of metallic Cu with bell pepper, at two different growth stages of the plant, indicated the antagonistic nature of the nano and micro sized Cu over the two exposure periods. Copper uptake and translocation was higher for bulk Cu treatments at the 45 day exposure period, whereas, it was higher for nano Cu treatments over the 90 day exposure period. The effect of weathering of the Cu treatments on the health of spinach plant was also in keeping with the initial hypothesis. The leaf health and nutrition in terms of carotenoid and chlorophyll were markedly hampered under the weathered CuO treatments.

For the very first experiment on the effects of nCuO, bCuO, and $CuCl_2$ on bell pepper plants, none of the treatments affected leaf chlorophyll in these plants. However, the gas exchange results showed that the ionic copper treatment had a negative effect on evapotranspiration, photosynthesis, and stomatal conductance. The agronomical parameters were not influenced by Cu treatments. None of the treatments reduced the uptake of essential elements in fruit. However, nCuO at 500 mg/kg significantly reduced Zn, compared with control. Overall, nCuO had an inhibitory effect on the uptake of important elements but did not show visible signs of toxicity, or

effects in plant productivity. Bulk CuO had stimulatory effects on the elemental nutrients in the plant tissue, contrarily.

Findings from the second experiment gave us clear evidence of the influence of particle size of metallic Cu on plant growth and Cu bioaccumulation at vegetative stage (VS) and reproductive stage (RS) in bell pepper plants. At the VS study, Cu uptake and bioaccumulation were significantly higher in the plant tissue (especially leaf) from bCu treatment exposure. For the RS study, conversely, the Cu bioaccumulation was significantly higher at 500 mg nCu/kg treatment in the root and leaf tissue. Gas exchange parameters were also significantly higher for the nCu treatments as compared to the bulk counterpart. No significant influence on the catalase and ascorbate peroxidase enzyme activity was seen in the leaf and root during the RS study, along with the fruit nutrient quality parameters (carotenoid, chlorophyll, total sugar, and vitamin C content). Some data indicate that over the short term exposure, 500 mg nCu/kg had stimulatory effects and could be used as a fertilizer/plant growth promoter.

The third experiment confirmed the hypothesis that weathering in soil would further enhance the negative influence of the Cu treatments on plants. Data from this experiment has shown that the effect of weathering of the (nano and bulk) CuO treatments are observable only in the aerial parts of spinach plants. However, unweathered bCuO treatment had significantly higher root catalase activity, compared to the weathered counterpart, showing an influence of the treatment, especially towards the end of the plant life cycle. In addition, weathering did not affect Cu bioaccumulation in the root or the leaves. However, the effect of weathering was evident in leaf pigments, chlorophyll *a*, and carotenoid. Weathered control and ionic treatments had significant ill effect on the foliar health of the plant. Significant deterioration of the two pigments

compared to control suggested that W CuO treatments exercised an influence on the plant foliar health at the mid and towards the end of the plant life cycle.

Thrust area of the current project was the implications of nanotechnology in agriculture, against the beneficial ENP industrial applications. This work finds its relevance in the fact that agricultural plants form an integral part of the human food chain. It also shows that ENPs could undergo possible bioaccumulation in plants and biomagnification in the food web. It is an attempt to add to the already existing databases of toxicants under surveillance to keep the food safety under check. Studies like these support formation of regulations on the yet unregulated ENPs. They could also support ENPs risk assessments and predictive toxicology tools.

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Annex I

This information is part of the discussion under chapter 2 of the dissertation.

Table A2.1. Root elemental concentration

Concentration of elements in the bell pepper root samples measured in mg of the essential element/kg of the dried sample. Plants were cultivated for 90 days in natural soil amended with Cu based chemicals at 0, 62.5, 125, 250, and 500 mg/kg concentrations. Statistically significant differences are being denoted by different letters between samples, as per the Tukey's test ($p \leq 0.05$). Concentration is an average of 4 replicates ± SE. Comparisons were made for one element at a time.

		nCuO	bCuO	$CuCl_2$
P	**Control**	2766.55 ± 196.36 a	2766.55 ± 196.36 a	2766.55 ± 196.36 a
	62.5 mg/kg	2012.87 ± 171.14 ab	2379.56 ± 70.05 ab	2679.21 ± 64.20 ab
	125 mg/kg	1937.15 ± 132.50 ab	2811.81 ± 294.37 ab	2086.57 ± 65.74 ab
	250 mg/kg	2070.44 ± 199.17 ab	2180.46 ± 85.08 ab	2634.74 ± 374.46 ab
	500 mg/kg	1919.39 ± 121.71 ab	1781.52 ± 138.55 b	2463.43 ± 270.95 ab
S	**Control**	2639.81 ± 167.81 b	2639.81 ± 167.81 b	2639.81 ± 167.81 b
	62.5 mg/kg	2332.20 ± 129.49 b	2888.01 ± 248.95 b	2741.03 ± 182.14 b
	125 mg/kg	2524.06 ± 168.61 b	2859.90 ± 120.62 b	2991.83 ± 270.92 ab
	250 mg/kg	2328.76 ± 140.98 b	2599.18 ± 65.87 b	2940.35 ± 146.32 ab
	500 mg/kg	2231.42 ± 209.86 b	2452.11 ± 95.26 b	4505.87 ± 1064.18 a
Se	**Control**	0.19 ± 0.11 b	0.19 ± 0.11 b	0.19 ± 0.11 b
	62.5 mg/kg	0.02268 ± 0.023 b	2.39237 ± 2.001 ab	0.919795 ± 0.920 b
	125 mg/kg	0.5989 ± 0.396 b	6.878 ± 2.34 a	0.924 ± 0.924 b
	250 mg/kg	0.170 ± 0.1699 b	3.52 ± 1.670 ab	0.043987 ± 0.05 b
	500 mg/kg	1.69 ± 1.0 ab	2.43 ± 1.335 ab	2.580 ± 2.043 ab
Mo	**Control**	2.83 ± 0.823 ab	2.83 ± 0.823 ab	2.83 ± 0.823 ab
	62.5 mg/kg	2.604 ± 0.54 ab	0	2.9 ± 0.46 ab
	125 mg/kg	1.60 ± 0.527 ab	0	4.87 ± 1.0055 a
	250 mg/kg	1.95 ± 0.52 ab	0	2.538 ± 0.2297 ab
	500 mg/kg	0.603 ± 0.28 b	0	3.05 ± 0.78 ab
Cu	**Control**	47.67 ± 2.01 c	47.67 ± 2.01 c	47.67 ± 2.01 c
	62.5 mg/kg	96.50 ± 11.70 bc	93.93 ± 6.0 bc	92.77 ± 10.98 bc
	125 mg/kg	102.07 ± 14.16 bc	85.61 ± 10.45 bc	127.40 ± 29.23 bc
	250 mg/kg	141.20 ± 10.42 b	135.57 ± 45.65 b	136.51 ± 100.50 b
	500 mg/kg	161.48 ± 117.51 b	118.73 ± 76.34 bc	256.31 ± 127.89 a

Table A2.2: Stem elemental concentration

Concentration of elements in the bell pepper stem samples measured in mg of the essential element/kg of the dried sample. Plants were cultivated for 90 days in natural soil amended with Cu based chemicals at 0, 62.5, 125, 250, and 500 mg/kg concentrations. Statistically significant differences are being denoted by different letters between samples, as per the Tukey's test ($p \leq 0.05$). Concentration is an average of 4 replicates ± SE. Comparisons were made for one element at a time.

		nCuO	**bCuO**	**$CuCl_2$**
S	**Control**	4223.59 ± 279.21 bc	4223.59 ± 279.214 bc	4223.59 ± 279.214 bc
	62.5 mg/kg	4333.158 ± 564.44 bc	4692.72 ± 846.66 abc	4144.46 ± 254.12 bc
	125 mg/kg	3351.12 ± 180.02 c	4107.61 ± 361.92 bc	4461.53 ± 331.34 bc
	250 mg/kg	3531.76 ± 353.71 c	4659.57 ± 503.14 abc	6769.61 ± 640.58 a
	500 mg/kg	3369.25 ± 209.04 c	3929.77 ± 107.08 c	6240.69 ± 672.15 ab
Zn	**Control**	15.21 ± 1.034 ab	15.21 ± 1.034 ab	15.21 ± 1.034 ab
	62.5 mg/kg	10.37 ± 0.358 ab	17.05 ± 4.4979 a	14.19 ± 0.859 ab
	125 mg/kg	7.88 ± 1.233 ab	13.19 ± 2.39ab	14.74 ± 2.009 ab
	250 mg/kg	8.62 ± 0.6459 ab	15.52 ± 1.67 ab	12.70 ± 2.56 ab
	500 mg/kg	7.11 ± 0.74 b	12.56 ± 1.2149 ab	10.56 ± 1.72 ab

Table A2.3: Leaf elemental concentration

Concentration of elements in the bell pepper leaf samples measured in mg of the essential element/kg of the dried sample. Plants were cultivated for 90 days in natural soil amended with Cu based chemicals at 0, 62.5, 125, 250, and 500 mg/kg concentrations. Statistically significant differences are being denoted by different letters between samples, as per the Tukey's test ($p \leq 0.05$). Concentration is an average of 4 replicates ± SE. Comparisons were made for one element at a time.

		nCuO	bCuO	$CuCl_2$
P	Control	3115.54 ± 121.87 ab	3115.54 ± 121.87 ab	3115.54 ± 121.87 ab
	62.5 mg/kg	2569.57 ± 217.10 ab	3640.06 ± 798.28 ab	3494.75 ± 128.15 ab
	125 mg/kg	2256.22 ± 112.07 b	3887.245 ± 506.83 a	3009.73 ± 168.41 ab
	250 mg/kg	2710.68 ± 167.20 ab	3356.70 ± 361.70 ab	3168.13 ± 186.12 ab
	500 mg/kg	2501.91 ± 137.63 ab	2929.86 ± 156.26 ab	3110.98 ± 4.874 ab
S	Control	4201.58 ± 120.895 b	4201.58 ± 120.895 b	4201.58 ± 120.895 b
	62.5 mg/kg	4179.03 ± 187.44 b	4523.07 ± 363.20 ab	4495.43 ± 185.92 ab
	125 mg/kg	3647.26 ± 149.00 b	4216.67 ± 249.61 b	4071.82 ± 175.37 b
	250 mg/kg	4051.73 ± 178.42 b	4276.00 ± 103.51 b	5865.53 ± 823.57 a
	500 mg/kg	4127.46 ± 122.5 b	3836.93 ± 349.27 b	4175.82 ± 254.84 b
R Mg	Control	10090.66 ± 320.379 b	10090.66 ± 320.380 a	10090.66 ± 320.379 a
	62.5 mg/kg	10270.49 ± 733.14 ab	10612.32 ± 841.623ab	11020.78 ± 223.48 ab
	125 mg/kg	10681.7 ± 761.570 ab	11121.33 ± 931.22 ab	11907.54 ± 477.66 ab
	250 mg/kg	10558.65 ± 878.28 ab	11425.45 ± 348.98 ab	11499.91 ± 296.13 ab
	500 mg/kg	11125.93 ± 557.41 ab	13078.47 ± 836.227 a	12704.06 ± 640.66 a
Se	Control	3.956 ± 1.005 ab	3.956 ± 1.005 ab	3.956 ± 1.005 ab
	62.5 mg/kg	1.25 ± 0.70 b	5.0333 ± 0.912 ab	1.445 ± 0.8996 b
	125 mg/kg	1.69 ± 0.587 b	8.9637 ± 2.218 a	0.80 ± 0.476 b
	250 mg/kg	1.13 ± 0.379 b	3.75 ± 0.968 ab	0.5088 ± 0.294 b
	500 mg/kg	0.88 ± 0.62 b	5.53 ± 1.686 ab	2.37 ± 1.215 b
Zn	Control	31.06 ± 2.88 a	31.06 ± 2.88 a	31.0589 ± 2.88 a
	62.5 mg/kg	17.42 ± 1.80 abc	28.93 ± 3.45 ab	30.84 ± 1.273 a
	125 mg/kg	15.97 ± 1.63 bc	28.9 ± 3.84 ab	24.23 ± 2.13 abc
	250 mg/kg	16.62 ± 1.11 abc	27.16 ± 2.43 abc	21.65 ± 1.73 abc
	500 mg/kg	13.95 ± 0.64 c	26.45 ± 0.92 abc	18.62 ± 2.54 abc
Cu	Control	11.30 ± 1.21 b	11.30 ± 1.21 b	11.30 ± 1.21 b
	62.5 mg/kg	17.88 ± 1.92 ab	17.88 ± 3.32 ab	19.79 ± 1.356 ab
	125 mg/kg	16.49 ± 1.11 ab	22.2897 ± 3.26711 a	20.1874 ± 2.88297 ab
	250 mg/kg	20.81 ± 1.80 ab	21.3892 ± 1.50597 a	25.8583 ± 2.15487 a
	500 mg/kg	21.17 ± 1.44 ab	20.7797 ± 0.83864 ab	24.4618 ± 1.80751 a

Table A2.4: Fruit elemental concentration

Concentration of elements in the bell pepper fruit samples measured in mg of the essential element/kg of the dried sample. Plants were cultivated for 90 days in natural soil amended with Cu based chemicals at 0, 62.5, 125, 250, and 500 mg/kg concentrations. Statistically significant differences are being denoted by different letters between samples, as per the Tukey's test ($p \leq 0.05$). Concentration is an average of 4 replicates ± SE. Comparisons were made for one element at a time.

		nCuO	bCuO	$CuCl_2$
P	**Control**	4499.16 ± 176.14 ab	4499.16 ± 176.14 ab	4499.16 ± 176.14 ab
	62.5 mg/kg	4243.46 ± 499.98 ab	4919.697 ± 203.74 a	4925.72 ± 482.01 a
	125 mg/kg	3923.18 ± 124.15 ab	5042.77 ± 326.038 a	4454.77 ± 302.37 ab
	250 mg/kg	3877.87 ± 208.63 ab	4656.89 ± 158.59 ab	4641.70 ± 303.59 ab
	500 mg/kg	3292.66 ± 130.14 b	4047.35 ± 147.997 ab	4614.67 ± 420.61 ab
Ca	**Control**	1326.83 ± 68.01 ab	1326.83 ± 68.01 ab	1326.83 ± 68.01 ab
	62.5 mg/kg	1322.74 ± 243.15 ab	1404.07 ± 69.87 ab	1439.03 ± 324.04 ab
	125 mg/kg	1247.51 ± 185.70 ab	1068.435 ± 30.56 ab	1352.40 ± 145.40 ab
	250 mg/kg	1212.29 ± 154.40 ab	1534.693 ± 87.75 ab	1104.66 ± 79.67 ab
	500 mg/kg	1026.36 ± 130.58 b	1314.35 ± 63.43 ab	1893.41 ± 360.38 a
Se	**Control**	0.41 ± 0.32 b	0.41 ± 0.32 b	0.41 ± 0.32 b
	62.5 mg/kg	0	1.113 ± 0.643 b	0.1996 ± 0.20 b
	125 mg/kg	0.26 ± 0.26 b	0.94 ± 0.76 b	0.11 ± 0.067 b
	250 mg/kg	0.20 ± 0.20 b	3.38 ± 0.67 a	0
	500 mg/kg	0.26 ± 0.26 b	1.06 ± 0.614 b	0.629 ± 0.28 b
Zn	**Control**	13.135 ± 0.88 abc	13.135 ± 0.88 abc	13.135 ± 0.88 abc
	62.5 mg/kg	10.88 ± 1.32 abcd	15.01 ± 0.44 a	12.38 ± 1.19 abc
	125 mg/kg	8.96 ± 0.54 bcd	13.48 ± 1.05 ab	10.31 ± 0.74 bcd
	250 mg/kg	8.33 ± 0.32 ab	14.37 ± 0.36 a	11.76 ± 0.46 abcd
	500 mg/kg	6.97 ± 0.296 d	11.40 ± 0.40 abcd	11.27 ± 1.30 abcd
Cu	**Control**	8.22 ± 0.32 b	8.22 ± 0.32 b	8.22 ± 0.32 b
	62.5 mg/kg	10.76 ± 1.21 ab	10.63 ± 0.52 ab	10.84 ± 0.51 ab
	125 mg/kg	10.70 ± 0.86 ab	10.56 ± 0.998 ab	9.35 ± 0.48 ab
	250 mg/kg	10.69 ± 0.81 ab	12.40 ± 0.84 a	11.80 ± 0.66 ab
	500 mg/kg	9.57 ± 0.83 ab	9.80 ± 0.58 ab	10.60 ± 0.93 ab
Ni	**Control**	0.23 ± 0.042 cd	0.23 ± 0.042 cd	0.23 ± 0.042 cd
	62.5 mg/kg	0.595 ± 0.05 abc	0.73 ± 0.11 a	0.45 ± 0.089 abcd
	125 mg/kg	0.20 ± 0.051 cd	0.713 ± 0.099 a	0.18 ± 0.061 d
	250 mg/kg	0.44 ± 0.129 abcd	0.68 ± 0.126 ab	0.44 ± 0.095 abcd
	500 mg/kg	0.52 ± 0.053 abcd	0.57 ± 0.058 abcd	0.28 ± 0.069 bcd

Table A2.5: Physical characteristics of the natural soil used in the study as analyzed by Hanna pH/EC/TDS/˚C meter (HI 9811-5)

	Soil sample in water	**Soil sample in $CaCl_2$**
pH	7.4	7.5
Electrical conductivity µS/cm	1740	3170
Total dissolved solid mg/L	860	1590

Table A2.6: The background concentration of elements in pristine soil used for experiments. The concentrations are averages of 3 replicates ± SE.

Element	**Concentration (mg/kg)**
K	3250.65 ± 92.60
S	398.48 ± 51.20
Mg	6723.40 ± 147.53
Ca	30317.15 ± 593.61
Fe	15133.04 ± 221.88
Zn	49.79 ± 0.68
Cu	17.34 ± 0.53
Mn	454.70 ± 8.33
Al	11791.76 ± 341.10
Cr	12.72 ± 0.33
Pb	20.53 ± 0.56
Ni	12.75 ± 0.25
P	835.21 ± 23.78
Si	1219.44 ± 38.37

Table A2.7: Translocation factors for Cu (TF = C_{leaf}/C_{root}), ratio of concentration of Cu in leaf vs that in root.

The TF are averages of 4 replicates ±SE.

Cu Species → Concentration ↓	Control	nCuO	bCuO	$CuCl_2$
Control	0.24±0.02			
62.5 mg/kg		0.20±0.04	0.20±0.05	0.22±0.03
125 mg/kg		0.17±0.03	0.26±0.03	0.18±0.04
250 mg/kg		0.15±0.02	0.18±0.04	0.19±0.02
500 mg/kg		0.14±0.02	0.18±0.02	0.10±0.02

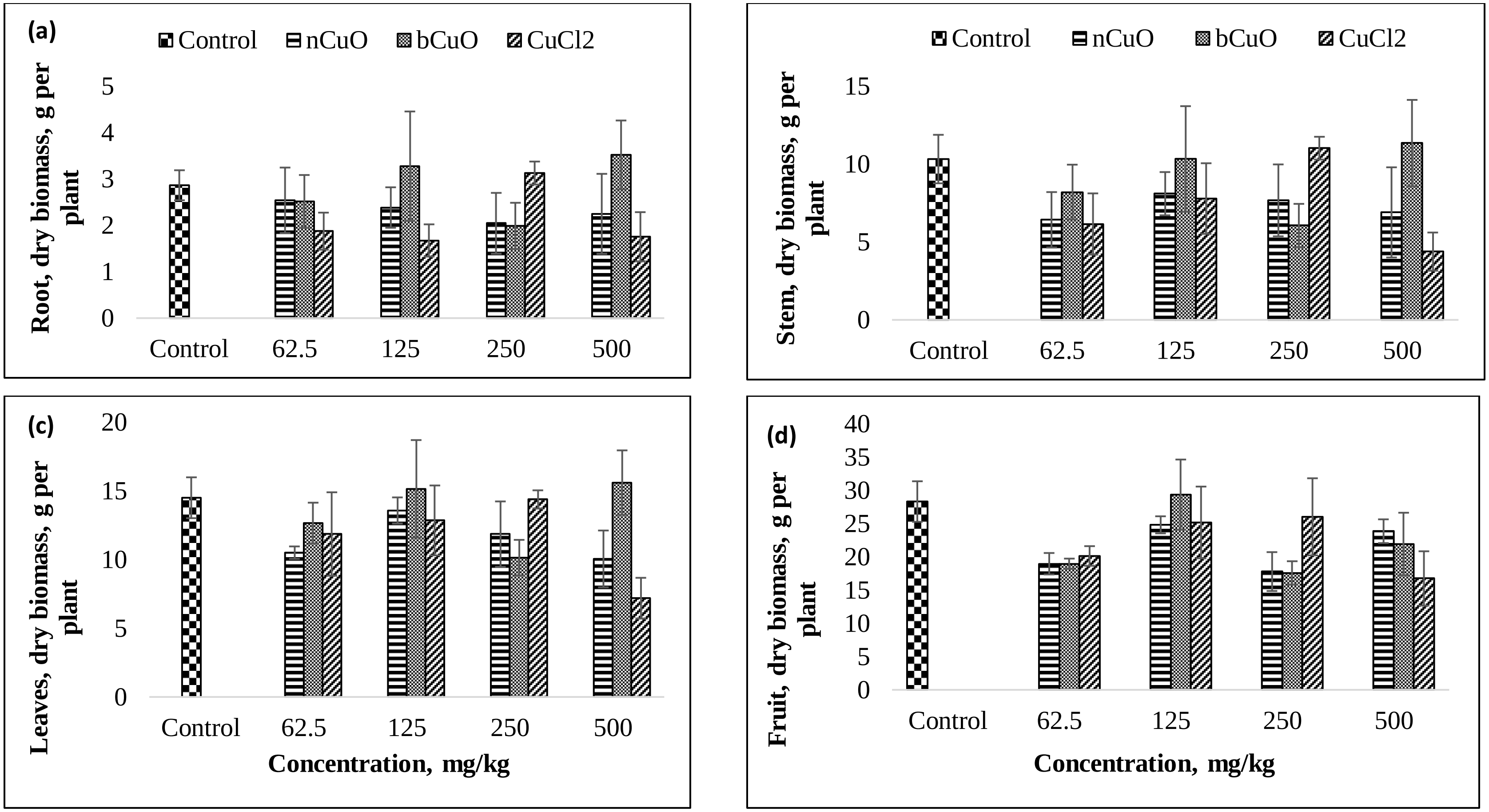

Figure A2.1: Plant biomass in (a) root (b) stem (c) leaves (d) fruit in bell pepper plants grown under the nCuO, bCuO, and $CuCl_2$ treatments. Data are averages of 4 replicates ± SE

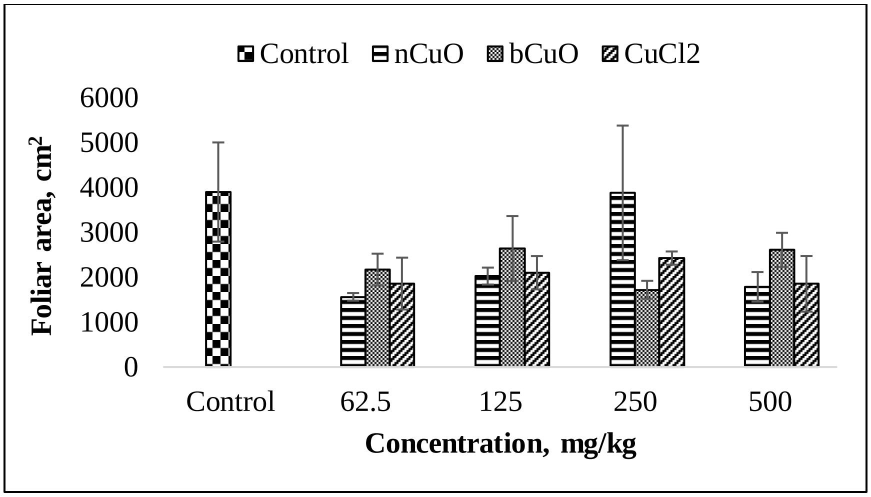

Fig. A2.2: Total foliar area in bell pepper plants grown under the nCuO, bCuO, and $CuCl_2$ treatments. Data are averages of 4 replicates ± SE.

Annex II

This information is part of the discussion under chapter 3 of the dissertation.

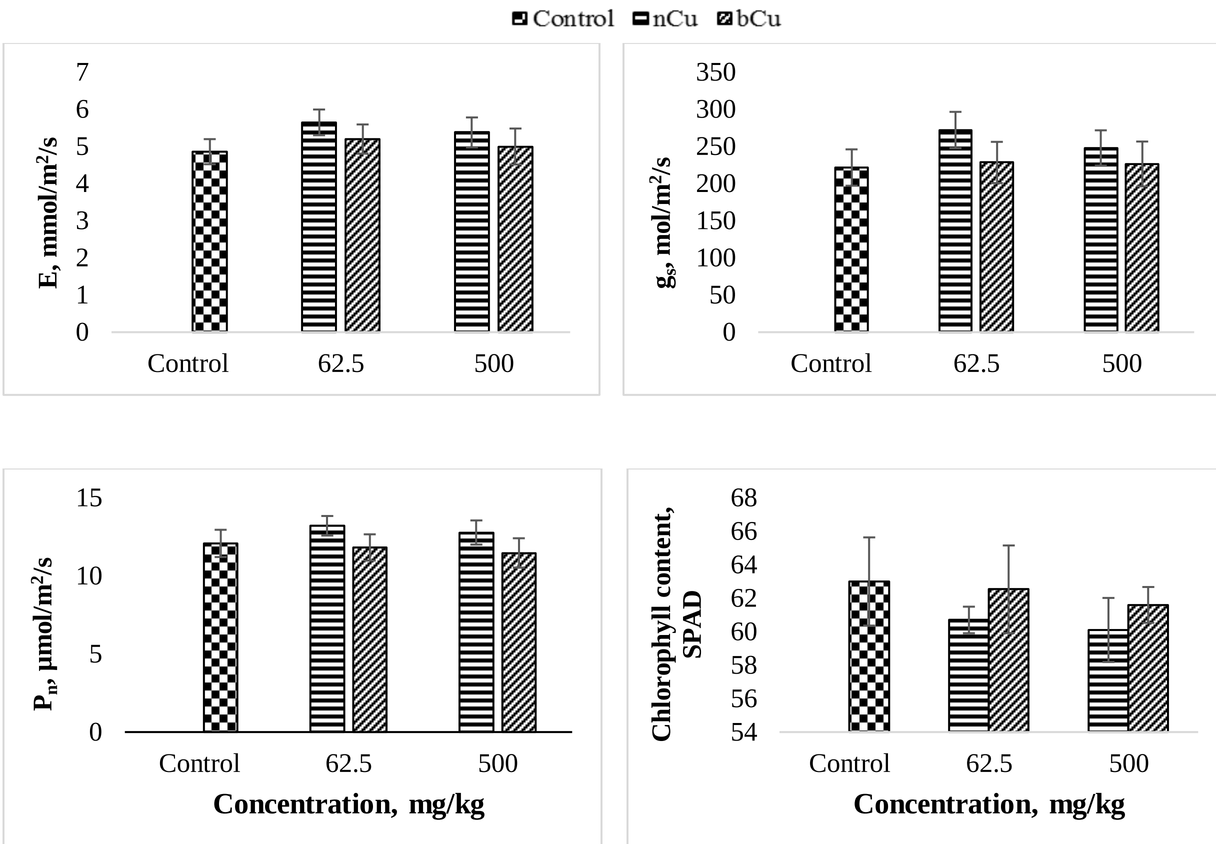

Figure A3.1: Gas exchange and SPAD measurements for the vegetative stage study (45 days post transplantation) in bell pepper plants grown under nCu and bCu treatments. Data are averages of 3 replicates ± SE ($p < 0.05$, $p < 0.1$)

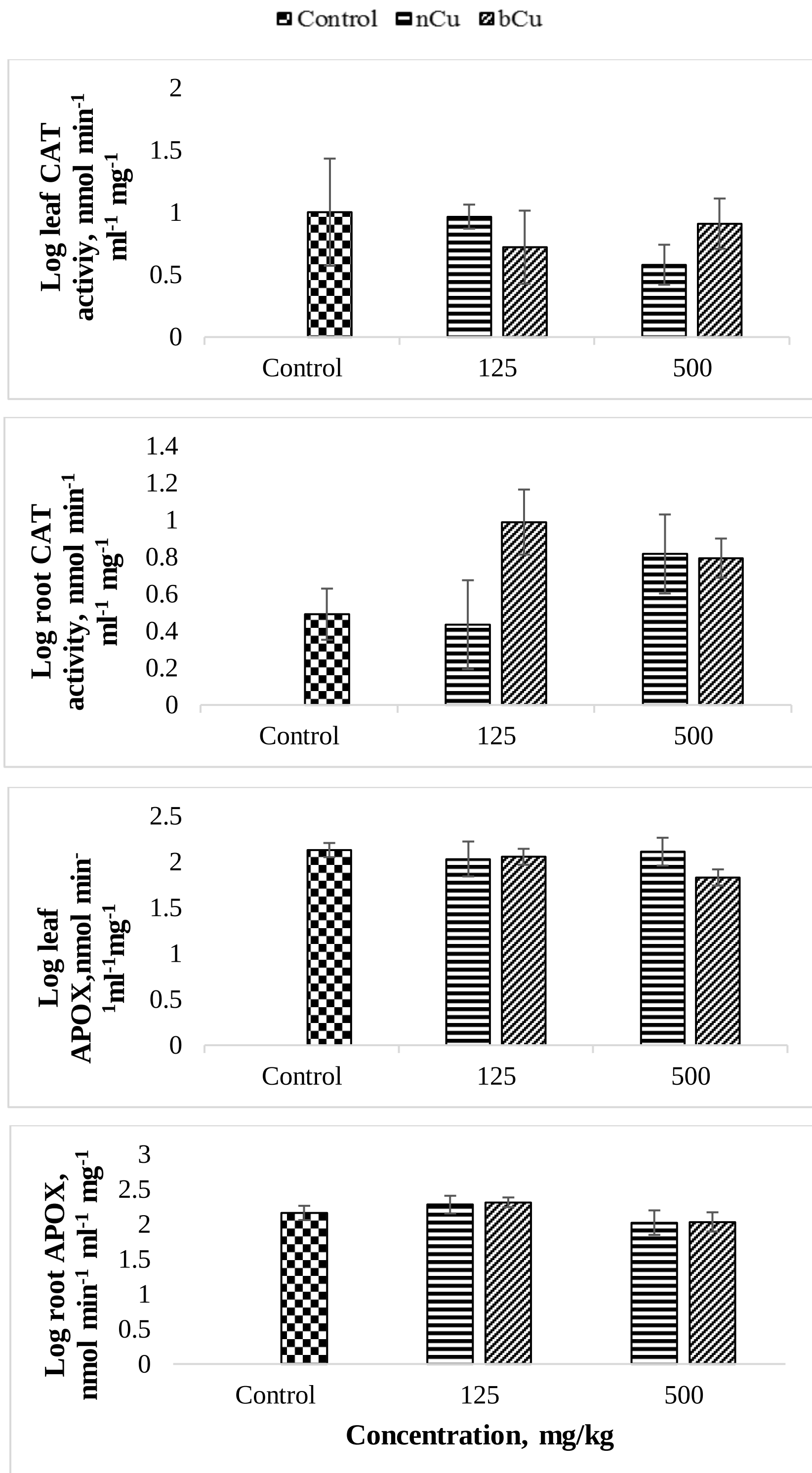

Figure A3.2: Log of catalase and ascorbate peroxidase activity at 75 days post transplantation in leaf and 150 days post transplantation in root of bell pepper plants grown under nCu and bCu treatments. Data are averages of 3 replicates ± SE ($p < 0.05$, $p < 0.1$)

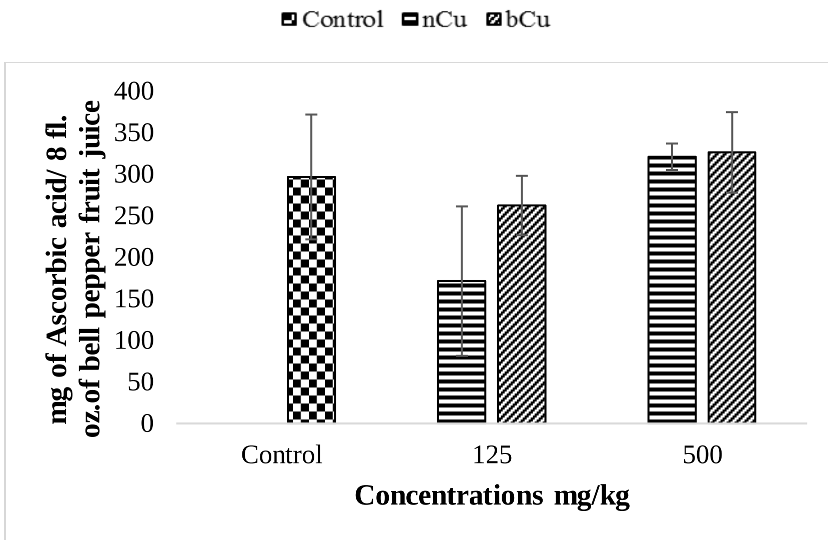

Figure A3.3: Vitamin C content at 75 days post transplantation in bell pepper fruits grown under nCu and bCu treatments during reproductive stage study. The data are averages of 3 replicates ± SE ($p < 0.05$, $p < 0.1$)

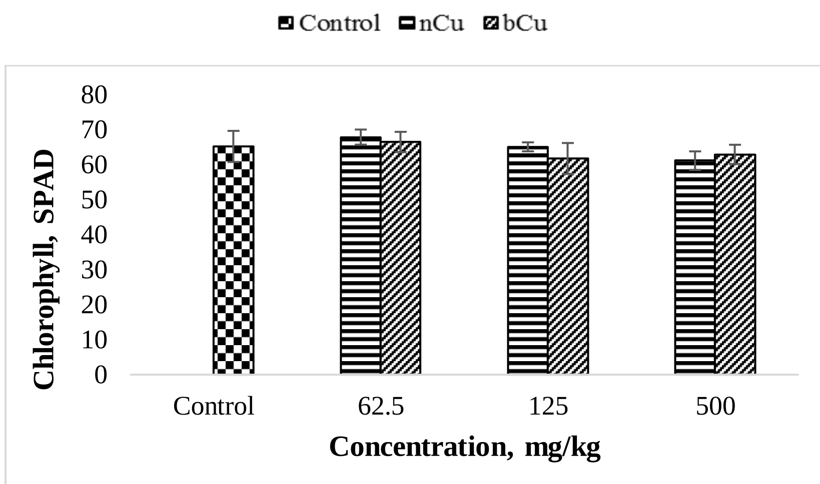

Figure A3.4: Chlorophyll (SPAD) measurements in bell pepper plants grown under nCu and bCu treatments at 90 days post transplantation for the reproductive stage study. Data are averages of 3 replicates ± SE ($p < 0.05$, $p < 0.1$)

Table A3.1. Major physicochemical properties of the Cu-based compounds used in this study (Hong et al., 2015; Lin et al., 2015)

Property	*n*Cu	Bulk Cu
Primary particle size (nm)	$10^2 - 10^3$	$< 10^4$
Hydrodynamic diameter (nm) [a]	2590±1138	4546±3940
Zeta potential (mV) [a]	-29.4±0.8	-35.4±1.27
Cu content (wt. %)	83.3	98.7
Other elements present	O, C	ND
Morphology	Irregular	Dendritic, plate-like, rhombus
Main Copper phase	Cu, Cu_2O	Cu
Crystal structure	Cubic	Cubic
purity	84.8 ± 2.7	94.9 ± 1.4

[a]Measurement was done at pH 7

ND- Non detect

Table A3.2: Physical characteristics of the natural soil used in the study as analyzed by Hanna pH/EC/TDS/˚C meter (HI 9811-5)

	Soil sample property
pH	7.4
Electrical conductivity µS/cm	1740
Total dissolved solid mg/L	860
Soil moisture content	3.55 ± 0.02 %
Soil organic matter content	2.11 ± 0.04 %

Table A3.3: The background concentration of elements in pristine soil used for experiments. The concentrations are averages of 3 replicates ± SE.

Element	Concentration (mg/kg)
K	3250.65 ± 92.60
S	398.48 ± 51.20
Mg	6723.40 ± 147.53
Ca	30317.15 ± 593.61
Fe	15133.04 ± 221.88
Zn	49.79 ± 0.68
Cu	17.34 ± 0.53
Mn	454.70 ± 8.33
Al	11791.76 ± 341.10
Cr	12.72 ± 0.33
Pb	20.53 ± 0.56
Ni	12.75 ± 0.25
P	835.21 ± 23.78
Si	1219.44 ± 38.37

Table A3.4: Pigments (µg/ml) at 75 days post transplantation in bell pepper fruit grown under nCu and bCu treatments during reproductive stage study. Data are averages of 3 replicates ± SE ($p < 0.05$, $p < 0.1$)

	Chlorophyll a	Chlorophyll b	Carotenoid
Control	0.61 ± 0.05	0.64 ± 0.04	0.15 ± 0.02
125 nCu	0.63 ± 0.02	0.70 ± 0.05	0.11 ± 0.03
500 nCu	0.67 ± 0.14	0.73 ± 0.14	0.25 ± 0.07
125 bCu	0.58 ± 0.05	0.60 ± 0.02	0.19 ±0.06
500 bCu	0.47 ± 0.03	0.59 ± 0.03	0.13 ± 0.02

Table A3.5: Total sugar (mg/ml) at 90 days post transplantation in bell pepper fruits grown under nCu and bCu treatments during reproductive stage study. Data are averages of 3 replicates ± SE ($p < 0.05$, $p < 0.1$)

	Total Sugar, mg/ml
Control	4.05 ± 0.35
62.5 nCu	3.07 ± 0.43
125 nCu	3.16 ± 0.23
500 nCu	4.25 ± 0.61
62.5 bCu	3.97 ± 0.66
125 bCu	3.23 ± 0.10
500 bCu	3.65 ± 0.48

Annex III

This information is part of the discussion under chapter 4 of the dissertation.

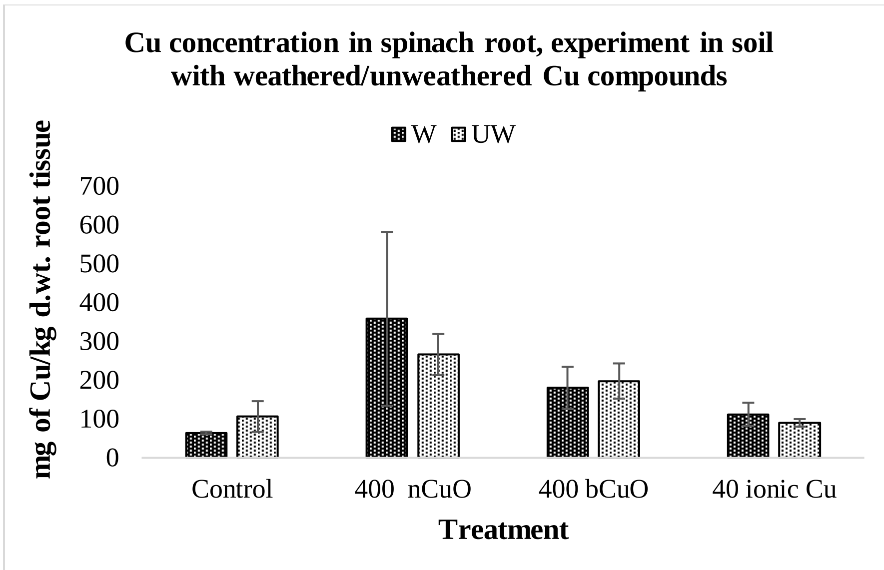

Figure A4.1: Comparison of the copper bioaccumulation in the spinach (*Spinacia oleracea)* root with plants grown in weathered vs unweathered control (0 mg/kg), 400 mg/kg nCuO, 400 mg/kg bCuO, 40 mg/kg ionic Cu treatments in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of 3 replicates ± SE. ANOVA followed by Tukey-Kramer multiple comparison test was performed on the data (p<0.05).

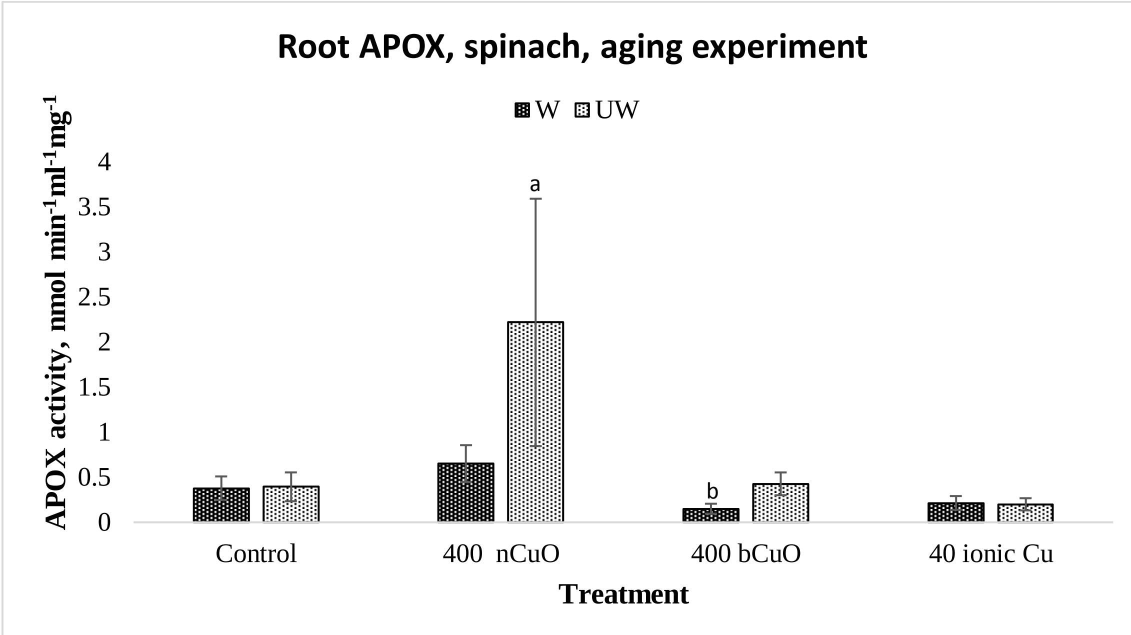

Figure A4.2: Comparison of the ascorbate peroxidase activity in spinach (*Spinacia oleracea)* roots measured at 45 days post transplantation. The plants were grown in weathered vs unweathered control (0 mg/kg), 400 mg/kg nCuO, 400 mg/kg bCuO, and 40 mg/kg ionic Cu treatments in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of 3 replicates ± SE. Average values with uncommon letters are statistically significantly different with respect to each other, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test ($p<0.05$).

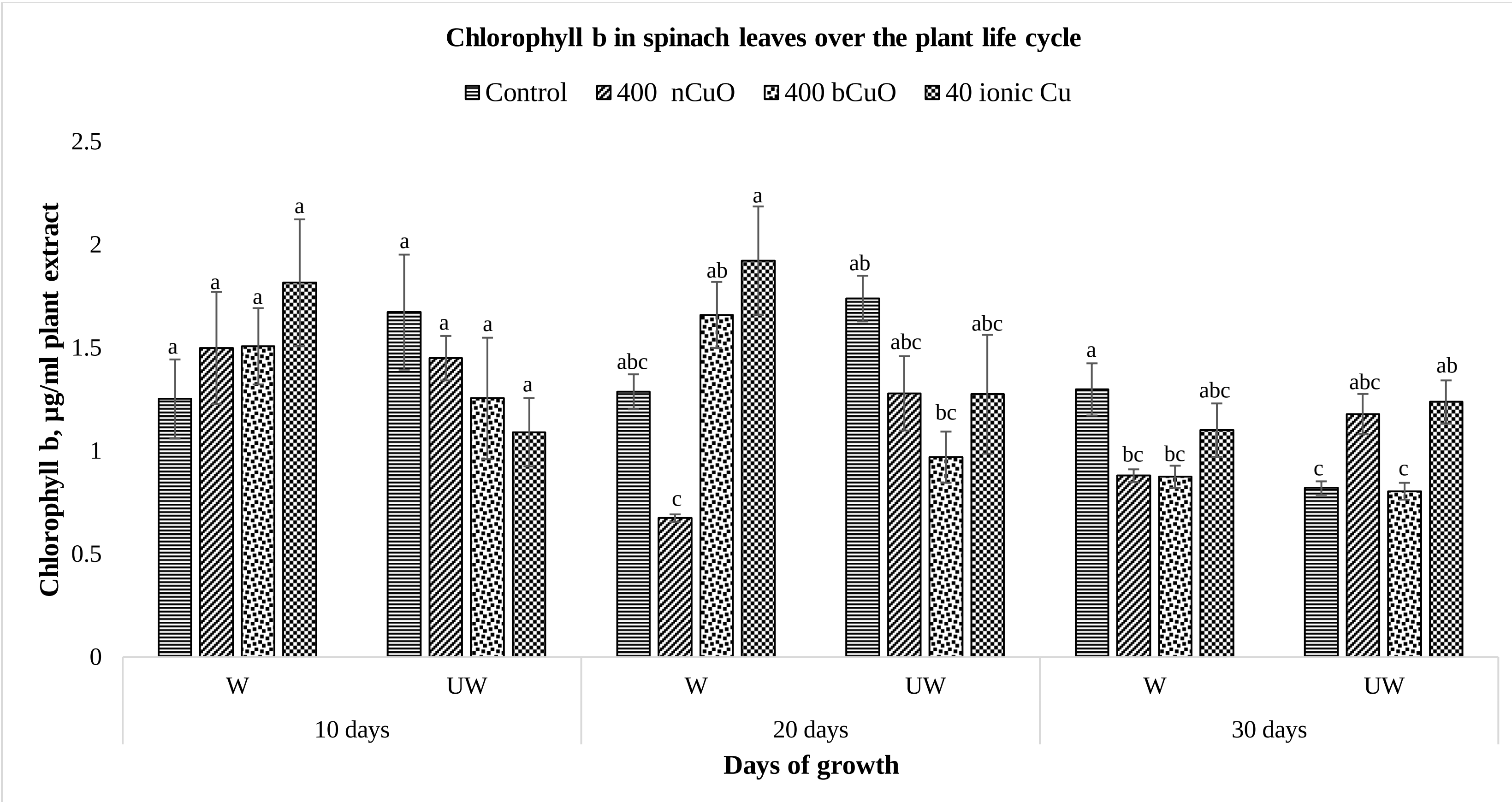

Figure A4.3: Comparison of the leaf chlorophyll b content at various time points during the growth cycle of spinach plants grown in weathered vs unweathered control (0 mg/kg), 400 mg/kg nCuO, 400 mg/kg bCuO, and 40 mg/kg ionic Cu treatments in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of 3 biological replicates and 3 subsequent technical replicates ± SE. Average values with uncommon letters are statistically significantly different with respect to each other, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test (p<0.05).

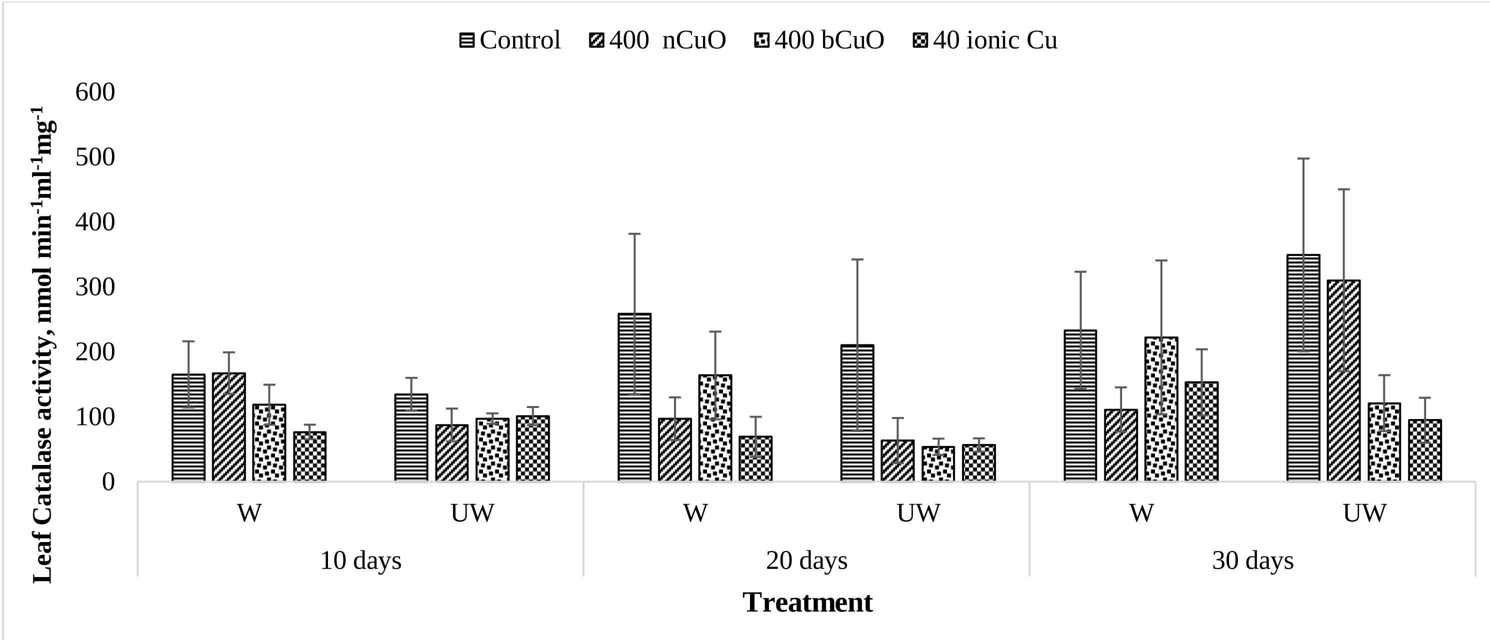

Figure A4.4: Comparison of the leaf Catalase activity at various time points during the growth cycle of spinach plants grown in weathered vs unweathered control (0 mg/kg), 400 mg/kg nCuO, 400 mg/kg bCuO, and 40 mg/kg ionic Cu treatments in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of 3 biological replicates and 3 subsequent technical replicates ± SE. Statistical analysis was done via one-way ANOVA followed by Tukey-Kramer multiple comparison test (p<0.05).

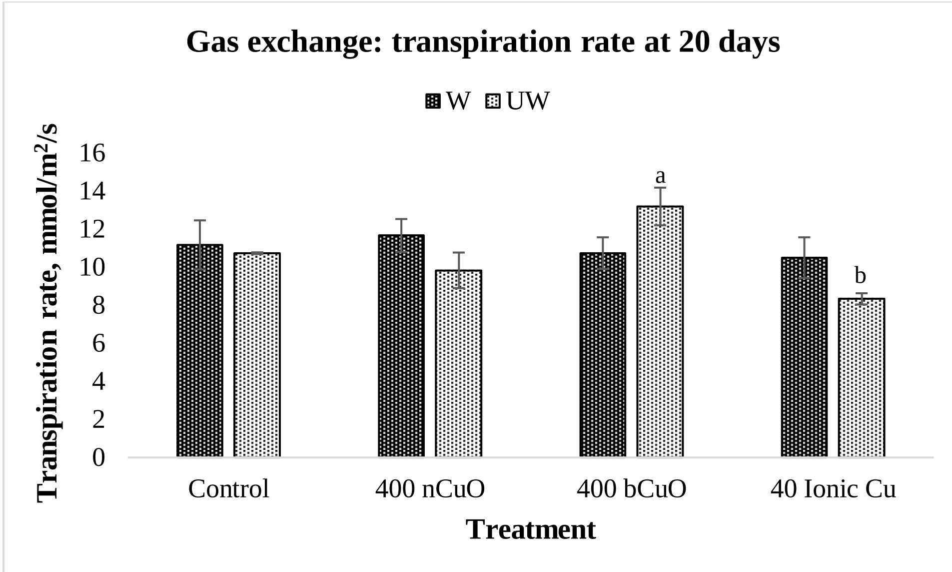

Figure A4.5: Comparison of the rate of transpiration in spinach (*Spinacia oleracea)* leaves measured at 20 days post transplantation. The plants were grown in weathered vs unweathered control (0 mg/kg), 400 mg/kg nCuO, 400 mg/kg bCuO, and 40 mg/kg ionic Cu treatments in soil (natural soil: potting mix:: 2:1) for 45 days under greenhouse conditions. Data are averages of 3 replicates ± SE. Average values with uncommon letters are statistically significantly different with respect to each other, as per one-way ANOVA followed by Tukey-Kramer multiple comparison test ($p<0.1$).

Table A4.1. Physico-chemical characteristics of nCuO and bCuO particles used in this study (Hong et al., 2015; Lin et al., 2015)

Property	**nCuO**	**bCuO**
Primary particle size (nm)	20 – 100	200– 2000
Hydrodynamic diameter (nm) [a]	280±15	376±26
Zeta potential (mV) [a]	-34.4±0.5	-42.7±0.153
Cu content (wt. %)	74.3	79.7
Purity (%)	88.3±1.3	92.8±1.1
Other elements present	O, C	O
Shape, Morphology	Rhombus, irregular	Prism, irregular
Main Copper phase	CuO	CuO
Crystal structure	Monoclinic	Monoclinic

Table A4.2: Ratio of chlorophyll a/ chlorophyll b. The numbers are averages of 3 replicates ± SE.

	10 days		20 days		30 days	
	W	**UW**	**W**	**UW**	**W**	**UW**
Control	0.8412 ± 0.0169 ab	1.18498 ± 0.1944 a	0.88797 ± 0.0170 ab	0.73073 ± 0.0228 c	1.04243 ± 0.0805 a	0.83894 ± 0.0247 bc
400 nCuO	0.79878 ± 0.0279 b	0.72306 ± 0.0162 b	0.92713 ± 0.02370 a	0.7879 ± 0.0447 bc	0.96257 ± 0.03204 ab	0.78737 ± 0.043675 bc
400 bCuO	0.82432 ± 0.06267 b	0.83524 ± 0.05941 b	0.7917 ± 0.0249 bc	0.84335 ± 0.0287 abc	0.92084 ± 0.03941 abc	0.91403 ± 0.03058 abc
40 ionic Cu	0.84474 ± 0.0546 ab	0.743796 ± 0.0137 b	0.85366 ± 0.0167 ab	0.84249 ± 0.0159 abc	0.95316 ± 0.0196 abc	0.77015 ± 0.03169 c

Table A4.3: Elemental concentrations (mg/kg) in different treated soil samples after the plant harvest. Concentrations are averages of 3 replicates ± SE. Comparisons are made element wise (downwards) using one-way ANOVA followed by Tukey-Krammer multiple comparison test. Uncommon alphabets denote a significant difference (p<0.05).

	↓	**Mg**	**Ca**	**Fe**	**Cu**	**Al**	**Ni**	**P**
1	**Control W**	12856.66 ± 52.34 ab	28040.73 ± 298.08 **a**	17999.84 ± 292.89 ab	21.39 ± 1.41 **c**	15542.9 ± 245.57 ab	24.07 ± 0.71 **b**	1040.05 ± 45.78 ab
2	**Control UW**	14891.57 ± 415.13 ab	27368.94 ± 895.27 ab	19294.57 ± 242.86 ab	21.50 ± 1.16 **c**	17344.35 ± 320.32 **a**	33.18 ± 2.45 ab	1198.28 ± 53.91 ab
3	**400 nCuO W**	14751.38 ± 738.66 ab	27938.54 ± 946.08 **a**	19846.28 ± 727.61 **a**	**543.19 ± 37.70 a**	17829.02 ± 651.94 **a**	32.89 ± 3.06 ab	938.71 ± 130.38 ab
4	**400 nCuO UW**	15983.12 ± 1657.67 **a**	28169.42 ± 228.05 **a**	19463.38 ± 190.51 ab	454.74 ± 27.44 **ab**	17245.63 ± 594.14 **a**	41.39 ± 2.54 **a**	910.44 ± 51.58 ab
5	**400 bCuO W**	12810.96 ± 192.84 ab	27482.88 ± 152.71 ab	18904.59 ± 292.59 ab	**388.83 ± 61.11 b**	17016.83 ± 484.33 ab	29.58 ± 4.86 ab	825.75 ± 91.88 **b**
6	**400 bCuO UW**	15172.82 ± 580.25 ab	27564.89 ± 484.48 ab	20116.31 ± 855.37 **a**	467.42 ± 27.13 **ab**	17849.79 ± 751.69 **a**	33.55 ± 3.62 ab	1091.86 ± 193.54 ab
7	**40 Ionic Cu W**	14093.04 ± 1294.34 ab	26008.87 ± 1365.05 ab	18661.57 ± 528.47 ab	55.09 ± 18.12 **c**	16644.16 ± 382.93 ab	29.92 ± 5.51 ab	988.79 ± 24.67 ab
8	**40 Ionic Cu UW**	14276.36 ± 666.97 ab	27228.8 ± 677.68 ab	19170.89 ± 942.60 ab	79.39 ± 7.55 **c**	17027.8 ± 710.70 ab	30.64 ± 4.92 ab	1111.6 ± 61.55 ab
9	**Plain soil W**	11977.69 ± 147.34 ab	27840.88 ± 1386.38 **a**	17014.14 ± 326.31 **b**	21.49 ± 1.03 **c**	15280.84 ± 336.71 ab	23.77 ± 1.03 **b**	1174.83 ± 78.76 ab
10	**Plain soil UW**	14574.74 ± 420.01 ab	23825.31 ± 371.87 **b**	18055.33 ± 521.84 ab	22.26 ± 0.86 **c**	16622.66 ± 765.90 ab	28.26 ± 1.34 ab	1283.35 ± 34.05 **a**
11	**Fresh soil**	11701.92 ± 1107.97 **b**	26259.37 ± 131.10 ab	16922.95 ± 535.32 **b**	18.67 ± 0.05 **c**	14408.80 ± 538.25 **b**	19.89 ± 2.08 **b**	1079.38 ± 32.25 ab

Table A4.4: Comparison of the background elemental composition of 3 variation of the plain soil (natural soil: potting mix:: 2:1). Comparison was performed within each element group. The concentrations (mg/kg) are averages of 3 replicates ± SE.

→	**Plain soil, weathered**	**Plain soil, unweathered**	**Fresh soil**
S	927 ± 190.93 b	726.66 ± 53.34 b	780.14 ± 34.50 b
Mg	11977.69 ± 147.34 b	14574.74 ± 420.02 b	11701.92 ± 1107.97 b
Ca	**27840.88 ± 1386.38 a**	**23825.31 ± 371.87 b**	26259.37 ± 131.10 ab
Fe	17014.14 ± 326.31 b	18055.33 ± 521.84 b	16922.95 ± 535.32 b
Zn	**63.64 ± 0.97 a**	**63.54 ± 1.86 a**	**56.23 ± 1.16 b**
Cu	21.49 ± 1.03 ab	**22.26 ± 0.86 a**	**18.67 ± 0.05 b**
Mn	487.07 ± 7.61 b	511.09 ± 10.35 b	491.03 ± 15.76 b
Al	15280.84 ± 336.71 b	16622.66 ± 765.90 b	14408.80 ± 538.25 b
Cr	64.7 ± 4.11 b	100.02 ± 14.88 b	58.99 ± 11.89 b
Pb	14.91 ± 1.25 b	14.54 ± 1.22 b	16.27 ± 0.36 b
Ni	23.77 ± 1.03 ab	**28.26 ± 1.34 a**	**19.89 ± 2.08 b**
P	1174.83 ± 78.76 b	1283.35 ± 34.06 b	1079.38 ± 32.25 b
Radial K	5505.88 ± 102.34 b	7178.67 ± 606.65 b	5426.32 ± 492.81 b
Radial Mg	11891.98 ± 386.82 b	15180.35 ± 1041.07b	10958.06 ± 1281.46 b
Radial P	982.59 ± 51.94 b	1083.74 ± 40.64 b	904.33 ± 16.26 b
Radial S	927.42 ± 186.44 b	736.17 ± 82.96 b	769.00 ± 28.09 b

Table A4.5: Physical characteristics of the natural soil used in the study as analyzed by Hanna pH/EC/TDS/˚C meter (HI 9811-5) and Malvern Zetasizer (Malvern, UK).

Property	Value
pH	6.9/7.0
Electrical conductivity µS/cm	2160 ± 270
Conductivity (from Zetasizer) mS/cm	0.0753 ± 0.000252
Total dissolved solid mg/L	1075 ± 135
Zeta potential mV	-18.1 ± 0.416
Soil moisture content	14.67 ± 0.2434%
Soil organic matter content	7.782 ± 0.2376 %
Electrophoretic mobility $m^2s^{-1}V^{-1}$	-1.421 ± 0.03398

Figure A4.6: Necrosis in leaves at 45 days of plant growth in the weathered control samples (left) compared to healthy leaves in unweathered samples(right).

Figure A4.7: Necrosis in leaves at 45 days of plant growth in the weathered ionic samples (left) compared to healthy leaves in unweathered samples(right).

Curriculum Vita

Swati Rawat earned her Bachelor of Technology degree in Agricultural Engineering from G.B. Pant University of Agriculture and Technology, India in 2007. She worked as a Graduate Engineer Trainee with Tractor and Farm Equipment Ltd., Chennai, India before she moved to the USA for higher education. She got her Master of Science in Environmental Science from Baylor University, Waco, Texas in 2011. Thereafter, she worked with the Office of Research and Development, US Environmental Protection Agency at Athens, GA as a Research Assistant. Soon after she joined UTEP for a PhD in Environmental Science and Engineering.

Dr. Rawat is a recipient of a UTEP Dodson Research Grant (travel support), Graduate School travel award, and Graduate Scholarship. She served as the Cultural Secretary (2014-2015) and as the President (2015-2016) of the Indian Student Association at UTEP.

Dr. Rawat has presented her research at several meetings including 2018 Gordon Research Conference on Nanoscale Science & Engineering for Agriculture & Food Systems and has published her work in journals like Environmental Science: Nano. While pursuing her degree, Dr. Rawat worked as a Teaching Assistant with the Department of Chemistry and Biochemistry. Her dissertation titled, "*Effects of unweathered or soil weathered copper-based nanoparticles and compounds on soil grown bell pepper and spinach plants*" was supervised by Dr. Jorge Gardea-Torresdey. She's actively seeking a postdoctoral position and wishes to work as a Research Scientist in the field of 'Nano-technology in Agriculture'.

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