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# Root system architecture, copper uptake and tissue distribution in soybean (*Glycine max* cv. *Kowsar*) grown in copper oxide nanoparticles (CuONPs) amended soil and implications to human nutrition

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## Highlights

1. CuONPs and $CuCl_2$ reduced root architecture in soybean.
2. Particle size and concentration influenced Cu uptake and tissue distribution.
3. Concentration-response relationships were linear for CuONPs and $Cu^{2+}$ ions.
4. Copper biouptake followed the order: roots > leaves > stem > seeds.
5. CuONPs or $CuCl_2$ treatments improved nutritional Daily Values (DV) for Cu in soybean seeds.

**Abstract:** Understanding potential uptake and biodistribution of engineered nanoparticles in soil-grown plants is imperative for toxicity and risk assessment considering the oral exposure of edibles by humans. Herein, we assessed potential influence of particle size (25, 50, and 250 nm) and concentration (0, 50, 100, 200, and 500 mg/kg-soil) of Copper oxide nanoparticles (CuONPs) on: (1) the root system architecture, and the physicochemical attributes of soil at the soil-root interface, (2) leading to Cu transport and accumulation in root, stem, leaf and seed in soybean (*Glycine max* cv *Kowsar*) grown for entire lifecycle of 120 days, and compared with soluble $Cu^{2+}$ ions and water-only controls, and (3) performed a comparative assessment of total seed Cu levels in soybean with other valuable food sources for Cu intake and discussed its human health implications. Our findings showed particle size- and concentration-dependent influence of CuONPs on Cu uptake and tissue distribution in root, stem, leaf and seed in soybean. Alterations in root architecture (root dry weight, root length, root volume, and root area) were dependent on the Cu compound type, Cu concentrations, and their interactions ($p<0.05$), except for root density. Concentration-response relationships for all three sized CuONPs, and $Cu^{2+}$ ions, were linear. CuONPs and $Cu^{2+}$ ions had inhibitory effects on root growth and development. Overall, soybean responses to smallest size CuONPs-25 nm were higher for all parameters investigated compared to two larger sized CuONPs (50 nm, 250 nm) or $Cu^{2+}$ ions. Cu uptake/bioaccumulation differed among soybean tissues in the order: root > leaf > stem > seed. Despite reduced root architecture and seed yield, our smallest size CuONPs-25 nm led to increased total seed Cu uptake compared to the larger sized CuONPs and $Cu^{2+}$ ions tested. Our findings also suggest that soil amendment by CuONPs, more so by the smallest size CuONPs-25 nm, could significantly improve nutritional Cu value in soybean seed as reflected by % Daily Values (DV), and are rated "Good" to "Very Good" according to the "World's Healthiest Foods" rating. However, until the potential toxicity and risk from consumption of soybean seed is characterized in humans, caution should be exercised when the Cu fortified seeds are used for daily human consumption when addressing Cu deficiency and associated illnesses, globally.

**Key words:** Metal oxide nanoparticles, Bioaccumulation, Recommended Dietary Allowances, Daily Values, Essential nutrients

**Introduction**

Soybean (*Glycine max*) is an important and economical legume cultivated worldwide for food and feed products (Singh et al., 2007; Mataveli et al., 2010). Soybean seeds contain 20% oil (Lee et al., 2019), about 35-40% protein, and a complete set of essential amino acids critical for improving human (Xu et al., 2020). Soybean is considered the best source of plant protein and a standard for other plant protein sources (Blair, 2008). It is also an excellent source of carbohydrates (35%) and necessary elements including, copper, zinc, calcium, magnesium, iron, manganese, and phosphorus (Mataveli et al., 2010). Further, it contains metabolites such as isoflavone, saponins, phytic acids, and oligosaccharides (Ososki and Kennelly, 2003; Sakai and Kogiso, 2008). Soybean, like other legumes, enables nitrogen fixation by establishing a symbiotic association with specific rhizobium bacterium (*Bradyrhizobium japonicum*) (Kanchana et al., 2016).

The world's population is growing rapidly and is forecasted to reach 9.6 billion by 2050 (UN, 2019), thereby increasing demand for agricultural production (70% for grain production by 2050) (FAO, 2019). On the other hand, due to limited arable lands and usable water resources, dramatically increasing the use of chemical fertilizers is a conventional approach to achieve increased food production and meet population demands, globally (Liu and Lal, 2015). Crops such as soybean need nutrients to grow and improve yield, and soil nutrient deficiency can significantly reduce $N_2$-fixation, growth, and performance in plants (Miransari, 2016). Macronutrients (N, P, K, Ca, Mg, and S) and micronutrients (Fe, Zn, Mn, and Cu) promote different morphological physiological functions in plants, including enzyme activities and oxidation-reduction processes (Miransari, 2016). Micronutrients can be supplemented to plants through chemical fertilizers (Welch and Graham, 2004). Interest in the use of nanofertilizers have recently increased due to their unique physicochemical characteristics not found in bulk or ionic counterparts (Servin et al., 2015; Raliya et al., 2017; Pokhrel et al., 2017; Kah et al., 2018). Micronutrients in nano-form may improve both yield and nutrient quality of crops compared to common micronutrients (ionic or bulk) conventionally delivered (Dimkpa and Bindraban, 2018). The response of plants to nanoparticles (NPs) depends on the chemistry of soil and nanoparticle, exposure dose, and species of crop (Pokhrel and Dubey, 2013, 2015; Dimkpa et al., 2019). Nanofertilizers may come in various forms: 1) nanofertilizers made of macro- and micro-nutrients; and 2) application of nanomaterials as nutrient carriers (Kah et al., 2018). Accordingly, the first group could supplement one or more nutrients for plants, and the second group could enhance the efficiency of conventional fertilizer delivery but does not directly supply nutrients to plants (Liu and Lal, 2015). Upon soil application, NPs can enter root, then penetrate the cell wall/plasma membrane, reaching the root cortex and enter the xylem vessels thereby moving up through the stele to aerial plant tissues (Ma et al., 2010; Rajput et al., 2019).

Cu is a redox metal that can exist in the form of cupric ($Cu^{2+}$/Cu (II)) and cuprous ($Cu^{+}$/Cu (I)) ions (Crowe et al., 2013). Cu (II) as anti-tumorigenic elicits cytotoxicity through cellular apoptosis (Chakraborty et al., 2010). In plants, Cu is a cofactor in a variety of proteins, including cytochrome C oxidase, plastocyanin, receptors of ethylene, and Cu/Zn-superoxide dismutase (Puig et al., 2007; Burkhead et al., 2009; Yuan et al., 2010). Cu-containing proteins are also involved in biological reactions such as Fe oxidation (Crowe et al., 2013), chlorophyll synthesis, and carbohydrate metabolism. About 70% of Cu is present in plant chloroplast (Rai et al., 2018). Biological activities including metabolism of cell wall and ethylene, photosynthesis, mitochondrial respiration, and protection against free radicals and oxidative stress, are disrupted with Cu deficiency (Puig et al., 2007; Burkhead et al., 2009; Yuan et al., 2010). Moreover, Cu deficiency could affect overall plant growth, and fruit and seed yield (Rai et al., 2018). Exposure to toxic levels of Cu could increase chlorosis, necrosis, root growth inhibition and an increase in lignin content, leading to reduced cell expansion and nutrient uptake (Lequeux et al., 2010; Finger-Teixeira et al., 2010; Shaw and Hossain, 2013; Miransari, 2016). Mixture of ionic and nanoparticles of Cu, Zn, and B were investigated, and mixed responses were observed for growth, yield and nutrient uptake in soybean (Dimkpa et al., 2017). Likewise, several studies have reported mixed results for Cu-NPs in lettuce, wheat and mung bean (Lee et al., 2008; Shah and Belozerova, 2009; Stampoulis et al., 2009).

Cu is naturally found in several foods and is available over the counter as a dietary supplement. Cu serves as a cofactor for many enzymes called cuproenzymes that play key roles in energy production, iron metabolism, neuropeptide activation, and synthesis of connective tissues and neurotransmitters in humans (IOM, 2001; Prohaska, 2012; Collins, 2014). Ceruloplasmin (CP), a cuproenzyme that constitutes over 95% of the total Cu in human plasma, plays a key role in iron metabolism (Hellman and Gitlin, 2002). Several physiologic processes including neurohormone homeostasis, angiogenesis, brain development, pigmentation, and regulation of gene expression and immune functioning are influenced by Cu (Collins, 2014). Cu-containing superoxide dismutases also play a major role in defense against oxidative damage (Owen, 1982; Allen and

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Klevay, 1994). According to the National Health and Nutrition Survey (NHANES) of data from 2009–2012, 6% to 15% of adults aged 19 and older who do not take dietary supplements containing Cu have Cu intake below the Estimated Average Requirement (EAR; an average daily intake estimated to meet the requirements of 50% of healthy individuals) (Blumberg et al., 2017). For adults using supplements, 2.2% to 7.2% of adults had intake below the copper EAR (Blumberg et al., 2017). Albeit uncommon, Cu deficiency could lead to anemia, hypopigmentation, hypercholesterolemia, connective tissue disorders, osteoporosis and other bone defects, abnormal lipid metabolism, ataxia, and increased risk of infection (Fairweather-Tait et al., 2011; Collins, 2014; Prohaska, 2014). It is therefore important to find ways to improve food Cu levels in edibles that are inexpensive and consumed widely.

Because soil-applied NPs will first come in contact with the root surfaces, following which biouptake and biodistribution of the elements (in pristine and/or modified form) could occur within the plant tissues, it is important to investigate potential effects of NPs on the root system architecture, soil-root interface, and potential accumulation in different tissues/organs in plants relative to its soluble ions, while building knowledge on ways to improve nutritional elements such as Cu in edible plant parts. Thus, in the present study, we assessed potential influence of particle size (25 nm, 50 nm, and 250 nm) and concentration (0, 50, 100, 200, 500 mg/kg-soil) of CuONPs on the root system architecture, and the physicochemical attributes of soil at the soil-root interface, leading to Cu transport and accumulation in root, stem, leaf and seeds in soybean, and compared with soluble $Cu^{2+}$ ions (positive control) and water-only control (negative control). A comparative assessment of total seed Cu levels in soybean with chickpeas and other valuable food sources for Cu intake and its implications to human health are also presented.

## Material and methods

### CuONPs synthesis and characterization

CuONPs with three different particle sizes (25 nm, 50 nm and 250 nm) were prepared by sol-gel method. The details of the synthesis protocol have been reported previously by our group (Yusefi-Tanha et al., 2020). Phase formation, crystal structure, microstructure and particle size distribution of the samples were characterized by X-ray diffraction (XRD) pattern analysis, and field emission scanning electron microscopy (FE-SEM). FE-SEM was also used for NP localization in the soybean seed. Dynamic light scattering (DLS) was used to estimate hydrodynamic diameter (HDD) and zeta potential of the CuONPs synthesized.

**Experimental set up**

Our experiment was carried out at the research farm of the Shahrekord University (50° 49 E, 32° 21´ N), Iran. The experiment was conducted using a factorial arrangement based on completely randomized design (CRD) with three replications. The treatments were $CuCl_2$ ($Cu^{2+}$; positive control) and three different sizes (CuONP; 25, 50, and 250 nm) and five concentrations (0, 50, 100, 200 and 500 mg CuONP or $Cu^{2+}$/kg). Each experimental unit consisted of two plants.

**Soil preparation and CuONPs exposure conditions**

Soil was collected from 0-30 cm depth and air dried for 7 days. It was sieved with a 2 mm sieve to separate any larger soil aggregates, wood chips and rocks. The percentage of sand, silt, and clay were 16%, 58%, and 26%, respectively. Additional properties of the soil are presented in **Table S1**. Based on soil test, fertilizers of urea (86 kg/ha, 46% N; as a starter) and triple superphosphate (100 kg/ha, 44% $P_2O_5$) were added before planting ensued. Soil pH and EC were continuously measured until harvest (at intervals of 30 days) following García-Gómez et al. (2018) in soil:water (1:5 suspensions). For soil amendment, copper compounds ($Cu^{2+}$ and CuONP 25 nm, 50 nm, and 250 nm) were suspended in 100 mL of distilled water to achieve desired concentrations (0, 50, 100, 200 and 500 mg CuONP or $Cu^{2+}$/kg-soil). Untreated soil was used as the negative control for each compound, and $CuCl_2$ was added to soil as the positive control. Each NP suspension was shaken (30 min, 25°C) before adding to the soil, then mixed with soil using a hand-mixer before sowing.

**Planting and crop management**

The present study was done under outdoor microcosm conditions for better simulation of the experimental conditions in the natural environment. Seeds of soybean (Kowsar cultivar) were procured from the Seed and Plant Improvement Institute, Karaj, Iran. Cultivation was done in polyethylene (PE) pots. Each pot contained 4 kg soil (control and amendment). For easier plant removal from pot at harvest, each pot had an inner liner of PE mesh (with 50 holes of 5 mm for drainage), which was filled with a layer washed gravel (500 g). After inoculation of soil by symbiotic bacteria *Rhizobium japonicum*, two seeds were planted at 2.5 cm depth about 24 h after amendment of soil. Irrigation was based on field capacity. A water sub-sample was evaluated for total Cu concentration using inductively coupled plasma-optical emission spectroscopy (ICP-OES) in each irrigation water. Upon maturity, the plants were harvested, collecting the whole plant. Aerial and roots tissues were then separated, oven dried (70°C for 48 h) in paper bags, weighed separately, and stored in plastic bags until analysis. Seeds were air-dried and stored.

**Measurement of root parameters**

To characterize the root system, root length (RL), root volume (RV), root area (RA), and root density (RD) were measured. After perfect washing the roots, a 1000 ml graduated cylinder was used to determine the root volume. So that a certain amount of water was poured into the cylinder, then the whole root was immersed in water and the volume added was equal to the root volume. The root length was measured by a ruler. Dry weight of root was taken and expressed in gram per plant. The root density was expressed by dividing the mean root dry weight by the root volume (De Baets et al., 2007):

$$RD = \frac{RDW}{RV} \quad (1)$$

where RDW (g) is mean root dry weight and RV ($cm^3$) is root volume.

The root area was calculated following the equation (De Baets et al., 2007):

$$RA = 2(RL \times \pi \times RV)^{0.5} \quad (2)$$

where RA ($cm^2$) is mean root area and RL (cm) is root length.

**ICP-OES analysis of total copper**

For measurement of total Cu accumulation in different plant tissues (root, stem, leaf, and seed), tissue samples (0.3 g) were washed several times with Milli-Q water and dried at 70°C for 48 h. Subsequently, they were digested with 10 mL $HNO_3$ (150°C for 1 h), then 2 mL $HClO_4$ at 215°C for 2 h (5:1 v/v). The digests were further diluted up to 10 mL using deionized water. The extracts were filtered before being analyzed using by ICP-OES (Ghasemi Siani et al., 2017).

**Seed copper concentration comparison with Recommended Dietary Allowance (RDA) and Daily Value (DV)**

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We compared soybean seed Cu levels with that in chickpeas seeds and other food sources. 100 g of soybean seed is assumed to be equivalent to 100 g of chickpeas seeds per serving of ½ cup, which is equivalent to 3.5 ounces. The Food and Nutrition Board (FNB) at the National Academies of Sciences, Engineering, and Medicine developed the Dietary Reference Intakes (DRIs) for Cu and other nutrients for human intake recommendations (NIH, 2020). DRI offers a set of reference values used for planning and assessing nutrient intakes of healthy people. Recommended Dietary Allowance (RDA) is an average daily intake considered sufficient to meet the nutrient requirements of almost all (97%–98%) healthy individuals; typically employed in planning diets that are nutritionally adequate for individuals (NIH, 2020). The U.S. Food and Drug Administration (FDA) developed Daily Values (DVs) to help consumers compare the nutrient contents in foods and dietary supplements within the context of a total diet. We used an RDA value of 0.9 mg (900 ug) for adults and children aged 4 years and older (US FDA, 2016) to calculate % DV (equivalent to % RDA) for soybean seeds grown under CuONPs or $Cu^{2+}$ ions treatments. Foods providing 20% or more of the DV are considered to be high sources of a nutrient. Next, we compared various Cu food sources from the U.S. Department of Agriculture, Agricultural Research Service's Food Data Central database (USDA, 2019). Finally, we classified our soybean seed Cu data based on the "World's Healthiest Foods" Rating following on the rule: Excellent, if DV ≥75%; Very Good, if DV ≥ 50%; Good, if DV ≥ 25% (http://www.whfoods.com/genpage.php?tname=foodspice&dbid=58).

**Statistical analysis**

A two-way analysis of variance (ANOVA) was performed using SAS (SAS Inc., ver. 9.4) to determine significant differences in crop responses to different treatments following a completely randomized experimental design. A Fisher LSD test at the 0.05 probability level was performed to further compare the means between the treatment groups. To determine if the concentration-response curves were linear (monotonic) or nonlinear (nonmonotonic), we coupled visual inspection of the curves with a simple decision rule: if the co-efficient of determination (R-squared) value for the linear regression line is 65% or higher the concentration-response curves were deemed linear, suggesting that the plant response changes linearly with the concentration applied following the relationship: $y = ax + b$; where y denotes dependent variable, x denotes independent

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variable, and a and b are model parameters. The computed R-squared values are presented in **Table 4**.

## Results and discussion

### Nanoparticle characterization

Despite apparent particle aggregation, the lognormal fitting of the particle size distribution using the FE-SEM micrographs showed that the mean particle size for S1, S2 and S3 samples are 25 nm, 50 nm and 250 nm, respectively (**Fig. 1**). The Reitveld analyses of the XRD patterns are shown in **Fig. 2** and the obtained structural parameters are summarized in **Table 1**. Our analyses indicate that the single phased CuONPs are crystallized in monoclinic structure in a highly pure form. All CuONPs samples are highly negatively charged with similar zeta potential (about -52 mV), but with different hydrodynamic diameters (189.0 nm, 195.1 nm, and 915.6 nm) (Yusefi-Tanha et al., 2020).

### Root dry weight

Our findings showed significant effects of copper compound type ($Cu_{type}$; $p$<0.0001), concentration (C; $p$<0.0001) and their interactions ($Cu_{type}$ × C) on root dry weight in soil-grown soybean ($p$≤0.01) (**Table 2**). Particle size- and concentration-dependent inhibition in root weight was observed upon exposure to CuONPs, and the two forms of Cu (CuONPs and $Cu^{2+}$) at all concentrations led to decreased root dry weight. However, the effect of CuONP-25 nm was significantly higher compared to the larger sized CuONPs or $Cu^{2+}$ ions treatments (**Table 3**). Root dry weight decreased linearly in a concentration-dependent fashion for the larger size CuONPs (CuONP-50, CuONP-250) and $Cu^{2+}$ ions whereas for CuONP-25 the relationships appeared nonlinear (**Table 4**). Although the lowest root dry weight was at 500 mg/kg CuONP-25 nm, it was not significantly different from 200 mg/kg CuONP-25 and 500 mg/kg CuONP-50 treatments. Compared with control, the root dry weight for plants exposed to CuONP-25 nm was reduced by 44.6%, 59.6%, 71.9 % and 82.75% for 50, 100, 200, and 500 mg Cu/kg treatments, respectively (**Table 3**). Control plants had the highest root dry weight (5.45 g/plant), and this was not significantly different from 50 mg/kg $Cu^{2+}$ treatment. In addition, plants treated with the larger sized CuONPs did not show significantly different root dry weight at all tested concentrations.

Also, average root dry weight was similar for CuONP-250 nm and $Cu^{2+}$ ions treatments up to 100 mg/kg (**Table 3**).

Although at low concentrations, Cu is a necessary micronutrient for plant growth and development, higher concentrations in soil-root interface could lead into harmful effects on plant growth (Nair and Chung, 2014a). Cu concentration in different plant tissues is typically in the range 2.0-50 µg/g dry weight (Barker and Pilbeam, 2015). Our results indicate particle size- and concentration-dependent toxicity of CuONPs in root weight of soybean, and that CuONPs toxicity may not be related to $Cu^{2+}$ ions released because $Cu^{2+}$ ions alone treatments resulted in significantly lower toxicity compared to CuONPs treatments at all concentrations (50-500 mg/kg) (**Table 3**). Previously, it was documented that exposure to CuONPs (<50 nm) could decrease stem and root growth in rice (Shaw and Hossain, 2013), barley (Shaw et al., 2014), and wheat (Dimkpa et al., 2012). Likewise, particle size- and concentration-dependent toxicity of CuONPs in *Arabidopsis* showed reduced root growth, roots lignification, and plant biomass (Nair and Chung, 2014b). Additionally, root lignification and growth modification in *Glycine max* (Lin et al., 2005) and *A. thaliana* (Lequeux et al., 2010) were reported with $Cu^{2+}$ exposure (0-5 µM), suggesting that absorbed dissolved Cu ions can lead to decreased root growth in soybean.

**Root length**

Significant effects of Cu compound type ($Cu_{type}$; $p$<0.0001), copper concentration (C; $p$<0.0001), and the interaction term $Cu_{type} \times C$ ($p$<0.01) were observed on soybean root length (**Table 2**). Our results show particle size- and concentration-dependent root length in soybean upon exposure to CuONPs, and CuONP-25 nm treatment led to smaller root length compared to the larger sized CuONPs and $Cu^{2+}$ ions treatments at most concentrations tested (**Table 3**). The concentration-response trend for CuONPs and $Cu^{2+}$ was linear (**Table 4**). Root length was, on average, 2 times lower upon 500 mg/kg CuONP-25 nm compared to control. Root length was similar at all concentrations tested for CuONP-50 nm and CuONP-250 nm (**Table 3**). The effects of CuONP-250 nm at 50 mg/kg was not significantly different from 50- and 100 mg $Cu^{2+}$/kg treatments and control ($p$<0.05).

The changes in plant root morphology upon exposure to CuONPs may point to a localized release of Cu in the form of NPs and/or ions upon contact between root cell surface and the NPs (Adams et al., 2016). Released Cu can alter meristematic activity and epidermal cell differentiation into root hairs (Adams et al., 2016). CuONPs (40-80 nm) were found to inhibit root elongation in maize (95.73%) and rice (97.28%) but at a higher concentration of 2000 mg/L (Yang et al., 2015). Our results for root length are consistent with the earlier studies that reported decreased root length in mustard (Nair and Chung, 2015), soybean (Nair and Chung, 2014a), and mung bean upon exposure to CuONPs (Nair et al., 2014).

**Root volume**

Root volume in soybean exposed to different Cu compounds are presented in **Table 5**. The results show that the effect of Cu compound type ($Cu_{type}$; $p$<0.0001), concentration (C; $p$<0.0001) and the interaction term ($Cu_{type}$ × C; $p \leq 0.05$) were statistically significant for root volume in soil-grown soybean (**Table 2**). Our findings clearly show changes related to particle size- and concentration in root volume upon exposure to CuONPs. Amongst all the treatments tested, root volume was significantly lowest at 500 mg/kg for CuONP-25 nm, but not significantly different with 200 mg/kg CuONP-25 nm and 500 mg/kg CuONP-50 nm, similar to root dry weight ($p$<0.05; **Table 5**). Further, root volume for CuONP-250 nm was similar to $Cu^{2+}$ ions treatment at all concentrations. At 50 mg/kg $Cu^{2+}$ ions, root volume was similar to control. For all CuONPs types, root volume was not significantly different at two lower concentrations (50 and 100 mg/kg), unlike two higher concentrations (200 and 500 mg/kg) (**Table 5**). The observed decrease in root volume upon exposure to small-sized CuONPs at higher concentrations could be attributed to decreased cell division, lateral root count, and root elongation (**Table 3**). Consistent to our study, a previous study also found reduced cell division and cell elongation, leading to reduced root elongation in sand-grown wheat upon treatment with CuONPs (>10 mg Cu/kg) (Adams et al., 2016).

**Root area**

ANOVA indicated that root area was affected by Cu compound type ($Cu_{type}$; $p$<0.0001), concentration (C; $p$<0.0001) and the interaction term ($Cu_{type}$ × C; $p$<0.001) (**Table 2**). Our results generally show particle size- and concentration-dependent root area in soybean upon exposure to CuONPs, and similar to other root parameters results (**Table 3, 5**), the decreasing effects of CuONP-25 nm was significantly higher compared to the larger sized CuONPs or $Cu^{2+}$ ions treatments for most concentrations (**Table 5**). The lowest root area was recorded at 500 mg/kg CuONP-25 nm that was not significantly different with CuONP-25 at 200 mg/kg (**Table 5**).

Further, at 50 mg/kg $Cu^{2+}$ ions, root area was not significantly different from control, akin to the root length and root volume (**Table 3, 5**). Also, there was no significant difference in root area between CuONP-250 nm and $Cu^{2+}$ ions at all concentrations tested ($p$<0.05). Small-sized CuONPs at higher concentrations decreased root area significantly due to reduced root length (**Table 3**) and root volume (**Table 5**). For CuONP-250 nm, the trend of root area change was relatively minimal across different concentrations tested.

**Root density**

Root density in soybean exposed to different copper compounds are presented in **Fig. 3A**. The effects of copper compound type ($Cu_{type}$; $p \leq 0.0001$) and concentration (C; $p<0.0001$) was significant on root density, but the interaction term ($Cu_{type} \times C$) was not ($p>0.5$; **Table 2**). Plants exposed to CuONP-25 nm showed the lowest root density compared to the larger sized CuONPs (50 and 250 nm) or $Cu^{2+}$ ions. Also, root density was similar in larger sized CuONPs (50 and 250) and $Cu^{2+}$ ions treatments (**Fig. 3A**). At the highest concentration of copper compounds (500 mg/kg), the root density was significantly reduced in comparison with the lower concentrations and control (**Fig. 3B**). In our study, CuONP-25 nm showed a significant decrease in root density with concomitant decrease in root dry weight (**Table 3**) and root volume (**Table 5**). In addition, with increasing concentration, the decrease in root density was greater compared to control (**Fig. 3B**).

**Root copper uptake**

The results indicated that the effects of Cu compound type ($Cu_{type}$), concentration (C) and the interaction term ($Cu_{type} \times C$) were significant for Cu concentration in soybean root ($p<0.0001$) (**Table 6**). Our findings show particle size- and concentration-dependent Cu uptake and accumulation in soybean root upon exposure to CuONPs during the lifecycle of 120 days. For all Cu compounds, root Cu concentration significantly increased compared to control, and the concentration-response curves for the larger size CuONPs (CuONP-50, CuONP-250) and $Cu^{2+}$ ions were linear (**Fig. 4A; Table 4**). Cu uptake was significantly higher for CuONP-25 nm compared to the larger sized CuONPs or $Cu^{2+}$ ions treatments at all concentrations tested. Compared to control, the average Cu concentration of root in plant treated with CuONP-25 nm at 50, 100, 200, and 500 mg/kg concentrations increased 1.7, 2.6, 3.0, and 3.3 times, respectively (**Fig. 4A**). The highest Cu concentration in soybean root at 500 mg/kg CuONP-25 nm that was not

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significantly different with CuONP-50 nm treatment at the same concentration. Also, the effects of CuONP-25 nm at 100 mg/kg was not significantly different from 200 mg/kg CuONP-50 nm treatment ($p<0.05$). Root Cu concentrations with 50 mg/kg of CuONP-25 nm treatment were similar to 100 mg/kg CuONP-50 nm and 200 mg/kg $Cu^{2+}$ ions treatments. In addition, at a concentration of 500 mg/kg the root Cu uptake upon CuONP-250 nm and $Cu^{2+}$ ions treatments were not statistically significant ($p<0.05$). As well as, there were no significant difference between 50 mg/kg larger sizes of CuONP (50 nm and 250 nm) and 100 mg/kg CuONP-250 and $Cu^{2+}$ ions ($p<0.05$) (**Fig. 4A**). Generally, root Cu uptake was similar for $Cu^{2+}$ ions and largest size CuONPs (250 nm), unlike the smallest size CuONPs (25 nm) that had highest root Cu uptake responses in soybean (**Fig. 4A**).

Several factors, including plant species, concentration used, root morphology, and soil properties, can influence Cu uptake and bioaccumulation in plants (Monica and Cremonini, 2009). Phytochelatins and metallothionein are momentous organic complexes, presumably formed in root cells, can promote Cu retention in the soil-root interface (Rawat et al., 2017). Cu ions from NPs have been shown to decrease root length, water content and dry biomass in lettuce. A significant accumulation of Cu in root exposed to Cu/CuONPs (20-30 nm) was documented compared to $CuSO_4.5H_2O$ in lettuce (Trujillo-Reyes et al., 2014). According to previous reports, Cu amount analysis indicated evidence for the presence of significant Cu amount in plant roots exposed to CuONPs. Likewise, Andreotti et al. (2015) found differences in Cu translocation in two salt marsh plant species (*Halimione portulacoides* and *Phragmites australis*). While Cu accumulated in the root of both plants, accumulation was significantly lower (4 - 10 times) when Cu was added as NPs. For *H. portulacoides*, no Cu translocation occurred in roots at 10 ppm NPs (<50 nm) treatment; whereas for *P. australis*, Cu translocation occurred regardless of the type of Cu used ($CuCl_2$ or CuONPs <50 nm) (Andreotti et al., 2015). In a recent study using cowpea, Ogunkunle et al. (2018) found a linear concentration-response upon exposure to CuNPs (< 25 nm and 60-80 nm) for Cu uptake by root, a finding consistent with our results. In Deng et al. (2020) study, the highest Cu concentration was found in Rosie variety of *Brassica rapa* plants treated with 600 mg/kg of CuONPs as their roots contained up to ~479 mg Cu/kg dry weight ($p \leq 0.05$).

**Stem copper uptake**

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A significant effect of Cu compound type ($Cu_{type}$; $p$<0.0001), concentration (C; $p$<0.0001) and the interaction term ($Cu_{type} \times C$; $p$<0.001) on stem Cu uptake in soybean was observed (**Table 6**). Our results show particle size- and concentration-dependent Cu uptake in soybean stem upon exposure to CuONPs. The concentration-response curves for all CuONPs and $Cu^{2+}$ ions were linear (**Table 4**). Exposure to CuONP-25 nm led to significantly higher Cu uptake in stem compared to the larger sized CuONPs and $Cu^{2+}$ ions treatments, and this uptake increased nearly two-fold at 500 mg/kg treatment compared to 50 mg/kg treatment for all Cu compound types (**Fig. 4B**). The stem Cu concentration increased to 7.3 mg/kg, on average, for CuONP-25 nm from the baseline of 3 mg/kg in control. Plants treated with CuONP-250 nm had similar stem Cu content compared to $Cu^{2+}$ ions treatment at all concentrations tested (**Fig. 4B**). Generally, stem Cu uptake was similar for $Cu^{2+}$ ions and larger size (50 nm and 250 nm) CuONPs, unlike the smallest size CuONPs (25 nm) that had highest stem Cu uptake responses in soybean (**Fig. 4B**).

Metal and metal oxide NPs can induce toxicity either by releasing toxic metal ions or by direct interaction with the cell (Manusadžianas et al., 2012; Andreotti et al., 2015). Plant can absorb metals as dissolved or soluble ionic fractions or absorb as NPs themselves. Dimkpa et al. (2012) observed Cu bioaccumulation in wheat stem upon exposure to 500 mg/kg CuONPs (<50 nm), and that soluble Cu from CuONPs was implicated in phytotoxicity. Consistent with our results, uptake of CuONPs by leaf fronds in *Landoltia punctata* was found to be more toxic with CuONPs treatments compared to ionic Cu treatments (Shi et al., 2011). Exposure to CuONPs (10-100 nm) and CuNPs (100-1000 nm) showed reduction in root growth in lettuce and alfalfa, and increased stem Cu content in alfalfa (Hong et al., 2015).

**Leaf copper uptake**

Our results show leaf Cu concentration in soybean was significantly affected by Cu compound type ($Cu_{type}$), concentration (C), and the interaction term ($Cu_{type} \times C$) ($p$<0.0001; **Table 6**). Further, leaf Cu uptake was particle size- and concentration-dependent. Similar to root Cu concentration, the concentration-response curves for the larger size CuONPs (CuONP-50 nm, CuONP-250 nm) and $Cu^{2+}$ ions were linear (**Fig. 4C**; **Table 4**). Leaf Cu concentration upon treatment with CuONP-25 nm at 50, 100, 200, and 500 mg/kg concentrations increased 3.9, 4.7, 5.7, and 6.0 times, respectively, compared to control. Leaf Cu uptake that was the highest at 500 mg/kg CuONP-25 nm treatment was not significantly different with 200 mg/kg CuONP-25 nm treatment.

Additionally, the effects of larger sized CuONPs (50 nm and 250 nm) were not significantly different when compared between 50 and 500 mg/kg treatments (**Fig. 4C**). Generally, leaf Cu uptake was similar for $Cu^{2+}$ ions and larger size (50 nm and 250 nm) CuONPs, unlike the smallest size CuONPs (25 nm) that had highest leaf Cu uptake responses in soybean (**Fig. 4C**).

Nanoparticles must be absorbed by the root for uptake and accumulation by aerial plant parts. They enter vascular tissue (xylem) upon penetrating the cell walls and plasma membrane, and translocating to stem, leaf and ultimately to seed (Ma et al., 2010). A linear relationship between Cu uptake/accumulation in different tissues and exposure concentrations of Cu-NPs in the growth medium was previously reported (Ma et al., 2010). The pores in cell wall could be below 10 nm in diameter (Ma et al., 2010; Albersheim et al., 2011), which is much smaller than the size range we tested for CuONPs. It was hypothesized that smaller sized ENP aggregates can pass through the pores reaching the plasma membrane, unlike larger aggregates that would not (Navarro et al., 2008 a,b; Kim et al., 2012). It is likely for ENPs to also create new pores upon cell surface interactions, enabling larger ENP internalization into plant tissues. However, cellular payloads such as innate and foreign macromolecules (e.g., proteins, peptides) could actively transport in and out of the cell (Ross et al., 2015), and studies have documented that such larger molecules might transport via plasmodesmata, a roughly cylindrical channel reaching up to 40 nm in diameter (Heinlein and Epel, 2004; Lucas and Lee, 2004; Microbe Notes, 2019).

Shi et al. (2014) found that exposure to CuONPs with diameter 34-52 nm (100, 200, 500, 1000, and 2000 mg/L) led to reduced root length, and NPs accumulation in root and leaf, in *Elsholtzia splendens*. Cu bioaccumulation in cowpea leaf was also enhanced by CuNPs with sizes < 25 nm and 60-80 nm; however, increasing exposure concentrations led to decreased translocation (Ogunkunle et al., 2018), suggesting a threshold for NP uptake and translocation in plants. Deng et al. (2020) also reported that leaf Cu accumulation pattern of *Brassica rapa* treated with nano CuO (75, 150, 300, and 600 mg Cu/kg soil) depends on particle size and plant phenotype. Our findings suggest that CuONPs with small size (25 nm) may be favored for cellular entry and translocation compared to the larger size CuONPs, promoting increase in leaf Cu uptake in soybean. Although dissolved, $Cu^{2+}$ ions showed overall lower uptake in different plant tissues compared to small size CuONPs (25 nm). In addition, the leaves of the soybean plant fall off after ripening or before harvesting. Therefore, biofortification of leaves in CuONPs treatments (**Fig. 4C**) may affect the microbial decomposition of soybean residues, and the positive and negative aspects of this subject need to be investigated in the future research.

**Seed copper uptake**

ANOVA showed that the effect of Cu compound type ($Cu_{type}$; $p<0.0001$), concentration (C; $p<0.0001$), and the interaction term ($Cu_{type} \times C$; $p<0.01$) were statistically significant for seed Cu concentration (**Table 6**). The results generally show particle size- and concentration-dependent seed Cu uptake in soybean upon exposure to CuONPs, and plant exposed to CuONP-25 nm typically showed higher Cu uptake compared to the larger sized CuONPs (50 nm and 250 nm) or $Cu^{2+}$ ions at most concentrations tested; a result consistent with the root, stem and leaf Cu uptake (**Fig. 4D**). The highest seed Cu concentration was observed at 500 mg/kg CuONP-25 nm, which was, on average, 1.8 times higher (6.55 mg/kg) compared to control. The concentration-response curves for CuONPs and $Cu^{2+}$ ions were linear, similar to other tissues examined. Seed Cu uptake at 50 mg/kg CuONP-25 nm treatment was not significantly different from 50 mg/kg CuONP-50 nm, 100 mg/kg of CuONP-250 nm, or 50 mg/kg $Cu^{2+}$ ions treatments ($p>0.05$). Similar to Cu concentration in soybean stem, average Cu concentration in soybean seed was similar between CuONP-250 nm and $Cu^{2+}$ ions at comparable concentrations. In addition, there was no significant difference in seed Cu uptake between CuONP-50 nm and $Cu^{2+}$ ions at 50, 200, and 500 mg/kg ($p<0.05$; **Fig. 4D**). Generally, seed Cu uptake was similar for $Cu^{2+}$ ions and larger size (50 nm and 250 nm) CuONPs, unlike the smallest size CuONPs (25 nm) that had highest seed Cu uptake responses in soybean (**Fig. 4D**).

Potential aggregation of CuONP-250 nm at higher concentrations may decrease metal bioavailability, reducing metal uptake and toxicity. In our study, total Cu concentrations differed among tissue types in the order: roots > leaves > stem > seeds (**Figs. 4A-D**), suggesting that the tissues furthest away from the root-soil system had the lowest total Cu (i.e., seed) and the root that is in direct contact with the soil had the highest total Cu levels. Wang et al. (2012) also showed metallic NPs phloem-based translocation from leaves to other parts of plant. CuONP-25 enabled higher Cu translocation to seed, probably due to its smaller size, less accumulation and easier passage through the cell wall. It was illustrated that NPs with smaller particle size can be increasing Cu availability even at low concentration, which may be due to increasing surface area of small nanoparticles (Andreotti et al., 2015). However, CuONPs with larger particle size can form more aggregates, reduce surface area and thus decrease availability of Cu, especially in the seed. Seeds of cowpea accumulated significant Cu amounts comparison to control, and the highest content of Cu was related to CuNPs < 25 nm and 60-80 nm, respectively at 500 mg/kg and 1000 mg/kg (Ogunkunle et al., 2018). In lettuce, Cu/CuONP treatments altered nutritional quality compared to the control, because this plant had more Cu, S, and Al, but less Mg, Ca, P, and Mn (Trujillo-Reyes et al., 2014).

**pH and EC of soil**

**Figs. 5A-B** demonstrates post-harvest soil pH change upon soil amended with various concentrations of different sized CuONPs and $CuCl_2$. Overall, the trends are similar with higher concentrations eliciting lower pH change than at lower concentrations for both NPs and ions of Cu.

Normal root activities including proton secretion, microbial activity at soil-root interface, and the potential release of root exudates can contribute to soil acidification to some degree (Adams et al., 2016; Dimkpa et al., 2018). Exposure to both compound types had similar soil pH change, suggesting similar mechanisms might be in play leading to increased $H^+$ ions concentrations. However, the presence of different organic and inorganic compounds capable of complexing Cu and changing its solubility may affect overall Cu bioavailability, independent of pH (Dimpka et al., 2018). Our findings indicate that during the growth period, pH of the soil reduced, but remained alkaline (**Figs. 5A-B**). At low pH, higher concentrations of NPs are more phytotoxic due to potential for more dissolution of metals (Qiu and Smolders, 2017). Accordingly, as CuONPs concentration increased, the available Cu increased with decreasing pH. Shi et al. (2014) found CuONPs dissolution promoted inside the cell because of the decreased cellular pH. In the present study with reducing pH as Cu concentrations increased in soil, a higher Cu uptake was associated with lower root growth (**Table 3, 5**), which could lead to decreased plant growth and yield in soybean (Yusefi-Tanha et al., 2020). **Fig. 5B** shows the pH changes for $CuCl_2$ amended soil at different concentrations, which were similar to the changes documented for CuONPs (**Fig. 5A**).

Copper is a low-mobility element in the soil, but can form very intense chelates (Rawat et al., 2017; Sekine et al., 2017). Potential toxicity of metal oxide NPs might be due to the dissolution and release of toxic ions. Dissolution can be affected by environmental conditions where the NPs are found; for example, pH and EC (Andreotti et al., 2015). Soil pH can affect metal bioavailability and phytotoxicity of NPs (García-Gómez et al., 2018). Generally, alkalinic condition promotes NPs aggregation, while acidic environment could trigger metal/oxide NPs dissolution, transforming into ionic species (Peretyazhko et al., 2014; Zhou et al., 2016). Additionally, in alkaline pH, aggregation of NPs can alter nano-specific attributes, and the dissolution propensity into ionic forms could decrease. However, with a pH change, the NPs can also disaggregate and return to a previous stable state. Likewise, NPs reactivity and toxicity can be modified with a minor change in surface charge and particle size (Silva et al., 2014), that this can also change as a function of media chemistry (Pokhrel et al., 2013; Pokhrel et al., 2014; Dimkpa, 2018).

**Figs. 5C-D** shows changes in post-harvest soil electrical conductivity (EC) under different sized CuONPs and $CuCl_2$ treatments. Overall, the trends are similar with higher concentrations eliciting lower EC change than at lower concentrations for both NPs and $Cu^{2+}$ ions. These results indicate lower root activities at higher concentration treatments leading to lower root exudate release into the soil-root interface and thus lower change in EC values.

**Implications for human nutrition**

The Food and Nutrition Board (FNB) at the National Academies of Sciences, Engineering, and Medicine have developed the Dietary Reference Intakes (DRIs) for copper and other nutrients for human intake recommendations (NIH, 2020). DRI offers a set of reference values used for planning and assessing nutrient intakes of healthy people. Recommended Dietary Allowance (RDA) is an average daily intake considered sufficient to meet the nutrient requirements of almost all (97%–98%) healthy individuals; typically employed in planning diets that are nutritionally adequate for individuals (NIH, 2020). The U.S. Food and Drug Administration (FDA) developed Daily Values (DVs) to help consumers compare the nutrient contents in foods and dietary supplements within the context of a total diet. The DV for Cu on the new Nutrition Facts and Supplement Facts labels and used for the values in **Table 7** is 0.9 mg (900 ug) for adults and children age 4 years and older (NIH, 2020). FDA required manufacturers to use these new labels starting in January 2020, but companies with annual sales of less than $10 million may continue to use the old labels that list a Cu DV of 2 mg (2,000 ug) until January 2021 (US FDA, 2013, 2017). The FDA does not require food labels to list Cu content unless it has been added to the food.

Foods that offers ≥20% of the DV are considered to be high sources of a nutrient, but foods providing lower percentages of the DV may also contribute to a healthful diet (NIH, 2020).

Our results of soybean seed Cu concentrations upon different sized CuONPs (25 nm, 50 nm, 250 nm) treatments at variable soil Cu concentrations (50-500 mg/kg soil) demonstrated the potential for significant improvement in seed Cu uptake (**Fig. 4D; Table 7**) with DV values in the range 44.0%–73.0%. For $Cu^{2+}$ ions, the DV values were in the range 44.2–67.0%. The highest DV values were recorded for the smallest size CuONPs-25 nm (DV=47.5%–73%), followed by CuONPs-50 nm (DV=45.6%–69.0%) and CuONPs-250 nm (DV=44.0%–65.0%). The DV values for ionic $Cu^{2+}$ mirrored that of largest size CuONPs-250 nm (**Table 7**).

Comparing soybean seed Cu concentrations with chickpeas seed, a legume considered good source of nutrients and phenolic compounds (e.g., polyphenols, isoflavones) with antioxidative potential to reduce oxidative effects with evidence supporting its consumption in prevention and management of diabetes and obesity (De Camargo et al., 2019), we found that our soybean seeds had 1.38–2.30 fold higher Cu concentrations than chickpeas seed per serving of ½ cup (100 g or 3.5 ounces) (**Table 7**). These results suggest that soil amendment by CuONPs, more so by smallest size CuONPs-25 nm could significantly improve nutritional Cu value in seed, and is found to be a better source of nutritional Cu compared to several other food items that provide nutritional Cu including Atlantic Salmon (wild, cooked), Avocado, Asparagus, Cream of Wheat, Dried Figs, Ground Turkey, Greek Yogurt, Non-fat Milk, Pasta, Sesame seeds, and Whole Wheat (**Table 7**) (NIH, 2020). Further, higher Cu soil amendment that led to higher seed Cu concentrations in soybean is comparable or higher than the food items which often provide higher DV of Cu including cooked mushroom (1/2 cup), cashew nuts (dry roasted, 1 ounce), cooked crab (Dungeness, 3 ounces), sunflower seed kernels (1/4 cup), simmered turkey giblets (3 ounces), dark chocolate (1 ounce), and raw tofu (1.2 cup) (NIH, 2020). Moreover, our soybean seeds were rated "Good" to "Very Good" according to the "World's Healthiest Food Ratings" based on the DV values (**Table 7**) (http://www.whfoods.com/genpage.php?tname=foodspice&dbid=58).

Because we found evidence of CuONPs within the cell wall, cell membrane and protein storage vacuoles in the cytoplasm of the soybean seed embryo using electron microscopy for CuONPs-25 nm treatment (**Fig. 6**), it is paramount to understand the potential toxicity of CuONPs in humans upon consumption of soybean seeds and oil. Future research should address what risk, if any, does

CuONPs pose to humans before such Cu fortified soybean seeds are used for daily human consumption to address Cu deficiency and related illnesses, globally.

## Conclusions

Utilization of CuONPs in modern agriculture as a novel fertilizer requires an understanding of their uptake, translocation and toxicity in plants. Our findings showed particle size- and concentration-dependent influence of CuONPs on Cu uptake and tissue distribution in root, stem, leaf and seed in soybean grown for full lifecycle of 120 days. Alterations in root architecture (root dry weight, root length, root volume, and root area) were dependent on the Cu compound type, Cu concentrations, and their interactions, except for the root density. Concentration-response relationships for all three sized CuONPs, including $Cu^{2+}$ ions, were found to be linear. CuONPs and $Cu^{2+}$ ions had inhibitory effects on root growth and development. Overall, soybean responses to the smallest size CuONPs-25 nm were higher for all parameters investigated compared to the two larger sized CuONPs (50 nm and 250 nm) or $Cu^{2+}$ ions. Cu uptake/bioaccumulation differed among soybean tissues in the order: root > leaf > stem > seed. Limited root growth could result in less plant utilization of water and soil nutrients, which can reduce plant growth and yield, and this is consistent with companion study showing particle-size and concentration dependent reduction in seed yield in soybean with CuONPs treatments (Yusefi-Tanha et al., 2020). Despite reduced root architecture and seed yield, our smallest size CuONPs-25 nm led to increased seed Cu uptake compared to other larger sized CuONPs and $Cu^{2+}$ ions tested. Our findings also suggest that soil amendment by CuONPs, more so by smallest size CuONPs-25 nm, could significantly improve nutritional Cu value in soybean seed as reflected by % Daily Values, and are rated "Good" to "Very Good" according to the "World's Healthiest Foods" rating. However, until the potential toxicity and risk from consumption of soybean seed is characterized in humans, caution should be exercised when the Cu fortified soybean seeds are used for daily human consumption when addressing Cu deficiency and related illnesses, globally.

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## Conflict of Interests

The authors declare no conflict of interest.

## Credit authorship contribution statement

Elham Yusefi-Tanha: Conceptualization, Investigation, Writing - original draft.

Sina Fallah: Conceptualization, Project administration, Supervision, Writing - original draft, Writing – review & editing, Funding acquisition.

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## References

Adams, J., Wright, M., Wagner, H., Valiente, J., Britt, D., Anderson, A., 2016. Cu from dissolution of CuO nanoparticles signals changes in root morphology. Plant Physiol. Biochem. https://doi: 10.1016/j.plaphy.2016.08.005.

Albersheim, P., Darvill, A., Roberts, K., Sederoff, R., Staehelin. A., 2011. Plant cell walls: from chemistry to biology. Garland Science, New York.

Allen, K.G., Klevay, L.M., 1994. Copper: an antioxidant nutrient for cardiovascular health. Curr. Opin. Lipidol. 5, 22-8.

Andreotti, F., Mucha, A.P., Caetano, C., Rodrigues, P., Gomes, C.R., Almeida, C.M.R., 2015. Interactions between salt marsh plants and Cu nanoparticles - Effects on metal uptake and phytoremediation processes. Ecotoxicol. Environ. Saf. 120, 303-309. http://dx.doi.org/10.1016/j.ecoenv.2015.06.017.

Barker, A.V., Pilbeam, D.J., 2015. Handbook of plant nutrition. CRC Press, Taylor & Francis Group, second edition. Pp, 774.

Blair, R., 2008. Nutrition and feeding of organic poultry, CAB International, ISBN 978-1-84593-406-4, Oxfordshire, England.

Blumberg, J.B., Frei, B., Fulgoni, V.L., Weaver, C.M., Zeisel, S.H., 2017. Contribution of dietary supplements to nutritional adequacy in various adult age groups. Nutr. 9 (12), 1325. https://doi.org/10.3390/nu9121325.

Burkhead, J.L., Reynolds, K.A.G., Abdel-Ghany, S.E., Cohu, C.M., Pilon, M., 2009. Copper homeostasis. New Phytol. 182, 799-816. https://doi.org/10.1111/j.1469-8137.2009.02846.x.

Chakraborty, A., Kumar, P., Ghosh, K., Roy, P., 2010. Evaluation of a Schiff base copper complex compound as potent anticancer molecule with multiple targets of action. Europ. J. Pharmacol. 647 (1-3), 1-12. https://doi.org/10.1016/j.ejphar.2010.08.003.

Collins, J.F., 2014. Copper. In: Ross, A.C., Caballero, B., Cousins, R.J., Tucker, K.L., Ziegler, T.R., (eds). Modern nutrition in health and disease. 11th ed. Baltimore, MD: Lippincott Williams & Wilkins. 206-216.

Crowe, S.A., Dossing, L.N., Beukes, N.J., Bau, M., Kruger, S.J., Frei, R., Canfield, D.E. 2013. Atmospheric oxygenation three billion years ago. Nature. 501, 535-538. https://doi.org/10.1038/nature12426.

De Baets, S., Poesen, J., Knapen, A., Barbera, G.G., Navarro, J.A., 2007. Root characteristics of representative Mediterranean plant species and their erosion-reducing potential during concentrated runoff. Plant Soil. 294, 169-183. https://doi.org/10.1007/s11104-007-9244-2.

De Camargo, A.C., Favero, B.T., Morzelle, M.C., Franchin, M., Alvarez-Parrilla, E., de la Rosa, L.A., Geraldi, M.V., Maróstica Júnior, M.R., Shahidi, F., Schwember, A.R., 2019. Is chickpea a potential substitute for soybean? phenolic bioactives and potential health benefits. Int. J. Mol. Sci. 20 (11), 2644. https://doi.org/10.3390/ijms20112644.

Deng, C., Wang, Y., Cota-Ruiz, K., Reyes, A., Sun, Y., Peralta-Videa, J., Hernandez-Viezcas, J.A., Turley, R.S., Niu, G., Li, C., Gardea-Torresdey, J., 2020. Bok choy (*Brassica rapa*) grown in copper oxide nanoparticles-amended soils exhibits toxicity in a phenotype-dependent manner: Translocation, biodistribution and nutritional disturbance. J. Hazard. Mater. 398, 122978. https://doi.org/10.1016/j.jhazmat.2020.122978.

Dimkpa, C.O., 2018. Soil properties influence the response of terrestrial plants to metallic nanoparticles exposure. Curr. Opin. Environ. Sci. Health. 6, 1-8. https://doi:10.1016/j.coesh.2018.06.007.

Dimkpa, C.O., Bindraban, P.S., 2018. Nanofertilizers: new products for the industry? J. Agric. Food Chem. 66 (26), 6462-6473. https://doi.org/10.1021/acs.jafc.7b02150.

Dimkpa, C.O., Bindraban, P.S., Fugice, J., Agyin-Birikorang, S., Singh, U., Hellums, D., 2017. Composite micronutrient nanoparticles and salts decrease drought stress in soybean. Agron. Sustain. Dev. 37, 5. https://doi.org/10.1007/s13593-016-0412-8.

Dimkpa, C.O., McLean, J.E., Latta, D.E., Manangón, E., Britt, D.W., Johnson, W.P., Boyanov, M.I., Anderson, A.J., 2012. CuO and ZnO nanoparticles: phytotoxicity, metal speciation, and induction of oxidative stress in sand-grown wheat. J. Nanopart. Res. 14, 1125. http://dx.doi.org/10.1007/s11051-012-1125-9.

Dimkpa, C.O., Singh, U., Adisa, I.O., Bindraban, P.S., Elmer, W.H., Gardea-Torresdey, J.L., White, J.C., 2018. Effects of manganese nanoparticle exposure on nutrient acquisition in wheat (*Triticum aestivum* L.). Agronomy. 8 (9), 158. https://doi:10.3390/agronomy8090158.

Dimkpa, C.O., Singh, U., Bindraban, P.S., Adisa, I.O., Elmer, W.H., Gardea-Torresdey, J.L., White, J.C., 2019. Addition-omission of zinc, copper, and boron nano and bulk oxide particles demonstrate element and size -specific response of soybean to micronutrients exposure. Sci. Total Environ. 665, 606-616. https://doi.org/10.1016/j.scitotenv.2019.02.142.

Fairweather-Tait, S.J., Harvey, L.J., Collings, R., 2011. Risk-benefit analysis of mineral intakes: case studies on copper and iron. Proc. Nutr. Soc. 70 (1), 1-9. https://doi.org/10.1017/S0029665110003873.

Finger-Teixeira, A., Ferrarese, M.dL.L., Soares, A.R., da Silva, D., Ferrarese-Filho, O., 2010. Cadmium-induced lignification restricts soybean root growth. Ecotoxicol. Environ. Saf. 73 (8), 1959-1964. https://doi.org/10.1016/j.ecoenv.2010.08.021.

Food and Agriculture Organization of the United Nations, 2019. http://www.fao.org/faostat/en/#data/QC.

García-Gómez, C., Obrador, A., González, D., Babín, M., Fernández, M.D., 2018. Comparative study of the phytotoxicity of ZnO nanoparticles and Zn accumulation in nine crops grown in a calcareous soil and an acidic soil. Sci. Total Environ. 644, 770-780. https://doi.org/10.1016/j.scitotenv.2018.06.356.

Ghasemi Siani, N., Fallah, S., Pokhrel, L.R., Rostamnejadi, A., 2017. Natural amelioration of zinc oxide nanoparticles toxicity in fenugreek (*Trigonella foenum-gracum*) by arbuscular mycorrhizal (*Glomus intraradices*) secretion of glomalin. Plant Physiol. Biochem. 112, 227-238. https://doi.org/10.1016/j.plaphy.2017.01.001.

Heinlein, M., Epel, B.L., 2004. Macromolecular transport and signaling through plasmodesmata. Int. Rev. Cytol. 235, 93-164.

Hellman, N.E., Gitlin, J.D., 2002. Ceruloplasmin metabolism and function. Annu. Rev. Nutr. 22, 439-58.

Hong, J., Rico, C.M., Zhao, L., Adeleye, A.S., Keller, A.A., Peralta-Videa, J.R., GardeaTorresdey, J.L., 2015. Toxic effects of copper-based nanoparticles or compounds to lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*). Environ. Sci. Process. Impact. 17, 177-185. https://doi.org/10.1039/c4em00551a.

Institute of Medicine, Food and Nutrition Board, 2001. Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington, DC: National Academies Press.

Kah, M., Kookana, R.S., Gogos, A., Bucheli, T.D., 2018. A critical evaluation of nanopesticides and nanofertilizers against their conventional analogues. Nat. Nanotechnol. 13, 677-684. https://doi.org/10.1038/s41565-018-0131-1.

Kanchana, P., Lakshmi Santha, M., Dilip Raja, K., 2016. A review on *Glycine max* (L.) Merr. (soybean). World J. Pharm. Pharm. Sci. 5 (1), 356-371.

Kim, S., Lee, S., Lee, I., 2012. Alteration of phytotoxicity and oxidant stress potential by metal oxide nanoparticles in *Cucumis sativus*. Water Air Soil Poll. 223 (5), 2799-2806. https://doi.org/10.1007/s11270-011-1067-3.

Lee, K., Rahman, M.S., Kim, A.N., Gul, K., Kang, S.W., Chun, J., Kerr, W.L., Choi, S.G., 2019. Quality characteristics and storage stability of low-fat tofu prepared with defatted soy flours treated by supercritical-$CO_2$ and hexane. LWT Food Sci. Technol. 100, 237-243. https://doi.org/10.1016/j.lwt.2018.10.073.

Lee, W.M., An, Y.J., Yoon, H., Kweon, H.S., 2008. Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*): plant agar test for water insoluble nanoparticles. Environ. Toxicol. Chem. 27, 1915-1921. https://doi.org/10.1897/07-481.1.

Lequeux, H., Hermans, C., Lutts, S., Verbruggen, N., 2010. Response to copper excess in *Arabidopsis thaliana*: impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. Plant Physiol. Biochem. 48 (8), 673-682. https://doi.org/10.1016/j.plaphy.2010.05.005.

Lin, C.C., Chen, L.M., Liu, Z.H., 2005. Rapid effect of copper on lignin biosynthesis in soybean roots. Plant Sci. 168 (3), 855-861. https://doi.org/10.1016/j.plantsci.2004.10.023.

Liu, R., Lal, R., 2015. Potentials of engineered nanoparticles as fertilizers for increasing agronomic productions. Sci. Total Environ. 514, 131-139. https://doi.org/10.1016/j.scitotenv.2015.01.104.

Lucas, W.J., Lee, J.Y., 2004. Plasmodesmata as a supracellular control network in plants. Nat. Rev. Mol. Cell Biol. 5, 712-726. https://doi.org/10.1038/nrm1470.

Ma, X., Geiser-Lee, J., Deng, Y., Kolmakov, A., 2010. Interactions between engineered nanoparticles (ENPs) and plants: phytotoxicity, uptake and accumulation. Sci. Total Environ. 408 (16), 3053-3061. https://doi.org/10.1016/j.scitotenv.2010.03.031.

Manusadžianas, L., Caillet, C., Fachetti, L., Gylytė, B., Grigutytė, R., Jurkonienė, S., Karitonas, R., Sadauskas, K., Thomas, F., Vitkus, R., Férard, J.F., 2012. Toxicity of copper oxide nanoparticle suspensions to aquatic biota. Environ. Toxicol. Chem. 31 (1), 108-114. https://doi.org/10.1002/etc.715.

Mataveli, L.R.V., Pohl, P., Mounicou, S., Arruda, M.A.Z., Szpunar, J., 2010. A comparative study of element concentrations and binding in transgenic and non-transgenic soybean seeds. Metallomics. 2, 800-805. https://doi.org/ 10.1039/C0MT00040J.

Microbe Notes. 2019. Available at: https://microbenotes.com/plasmodesmata-structure-and-functions/.

Miransari, M., 2016. Environmental stresses in soybean production. Elsevier, ISBN 978-0-12-801535-3, AbtinBerkeh Scientific Ltd. Company,Isfahan, Iran. pp, 273-298.

Monica, R.C., Cremonini, R., 2009. Nanoparticles and higher plants. J. Caryologia. 62 (2), 161-165. https://doi.org/10.1080/00087114.2004.10589681.

Nair, P.M.G., Chung, I.M., 2014a. A mechanistic study on the toxic effect of copper oxide nanoparticles in soybean (*Glycine max* L.) root development and lignification of root cells. Biol. Trace Elem. Res. 162 (1-3), 342-352. https://doi.org/10.1007/s12011-014-0106-5.

Nair, P.M.G., Chung, I.M., 2014b. Impact of copper oxide nanoparticles exposure on *Arabidopsis thaliana* growth root system development root lignificaion and molecular level changes. Environ. Sci. Pollut. Res. 21 (22), 12709-12722. https://doi.org/10.1007/s11356-014-3210-3.

Nair, P.M.G., Chung, I.M., 2015. Study on the correlation between copper oxide nanoparticles induced growth suppression and enhanced lignification in Indian mustard (*Brassica juncea* L.). Ecotoxicol. Environ. Saf. 113, 302-313. https://doi.org/10.1016/j.ecoenv.2014.12.013.

Nair, P.M.G., Kim, S.H., Chung, I.M., 2014. Copper oxide nanoparticle toxicity in mung bean (*Vigna radiata* L.) seedlings: physiological and molecular level responses of in vitro grown plants. Acta Physiol. Plant. 36, 2947-2958. https://doi.org/10.1007/s11738-014-1667-9.

National Institute of Health (NIH), 2020. Copper: Fact sheet for health professionals. Available at: https://ods.od.nih.gov/factsheets/Copper-HealthProfessional/#en3.

Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A.J., Quigg, A., Santschi, P.H., Sigg, L., 2008a. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicol. 17 (5), 372-386. https://doi.org/10.1007/s10646-008-0214-0.

Navarro, E., Piccapietra, F., Wagner, B., Marconi, F., Kaegi, R., Odzak, N., Sigg, L., Behra, R., 2008b. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. Environ. Sci. Technol. 42 (23), 8959-8964. https://doi.org/10.1021/es801785m.

Ogunkunle, C.O., Jimoh, M.A., Asogwa, N.T., Viswanathan, K., Vishwakarma, V., Fatoba, P.O., 2018. Effects of manufactured nano-copper on copper uptake, bioaccumulation and enzyme activities in cowpea grown on soil substrate. Ecotoxicol. Environ. Saf. 155, 86-93. https://doi.org/10.1016/j.ecoenv.2018.02.070.

Ososki, A.L., Kennelly, E.J., 2003. Phytoestrogens: a review of the present state of research. Phytother. Res. 17 (8), 845-869. https://doi.org/10.1002/ptr.1364.

Owen, C.A.J., 1982. Biochemical aspects of copper: copper proteins, ceruloplasmin, and copper protein binding. Park Ridge, NJ: Noyes Publications. Pp, 205.

Peretyazhko, T.S., Zhang, Q., Colvin, V.L., 2014. Size-controlled dissolution of silver nanoparticles at neutral and acidic pH conditions: kinetics and size changes. Environ. Sci. Technol. 48 (20), 11954-11961. https://doi.org/10.1021/es5023202.

Pokhrel, L.R, Dubey, B., 2015. Early developmental responses of plants exposed to metals and oxides nanomaterials. In: Siddiqui, M., Al-Whaibi, M., Mohammad, F., (eds). Nanotechnology and Plant Sciences. Springer, Cham. 153-164. https://doi.org/10.1007/978-3-319-14502-0_8.

Pokhrel, L.R., Bacon, C., Barbour, L., 2017. Nanotechnology-enabled agriculture is the future? EC Nutr. 9.4, 162-167.

Pokhrel, L.R., Dubey, B., 2013. Evaluation of developmental responses of two crop plants exposed to silver and zinc oxide nanoparticles. Sci. Total Environ. 452-453, 321-332. https://doi.org/10.1016/j.scitotenv.2013.02.059.

Pokhrel, L.R., Dubey, B., Scheuerman, P.R., 2013. Impacts of select organic ligands on the colloidal stability, dissolution dynamics and toxicity of silver nanoparticles. Environ. Sci. Technol. 47 (22), 12877-12885. https://doi.org/10.1021/es403462j.

Pokhrel, L.R., Dubey, B., Scheuerman, P.R., 2014. Natural water chemistry (dissolved organic carbon, pH, and hardness) modulates colloidal stability, dissolution kinetics and antimicrobial activity of silver nanoparticles. Environ. Sci.: Nano. 1, 45-54. https://doi.org/10.1039/C3EN00017F.

Prohaska, J.R., 2012. Copper. In: Erdman, J.W., Macdonald, I.A., Zeisel, S.H., (eds). Present knowledge in nutrition. 10th ed. Washington, DC: Wiley-Blackwell. 540-553.

Prohaska, J.R., 2014. Impact of copper deficiency in humans. Ann. N.Y. Acad. Sci. 1314 (1), 1-5. https://doi.org/10.1111/nyas.12354.

Puig, S., Andrés-Colás, N., García-Molina, A., Peñarrubia, L., 2007. Copper and iron homeostasis in Arabidopsis: responses to metal deficiencies, interactions and biotechnological applications. Plant Cell Environ. 30 (3), 271-290. https://doi.org/10.1111/j.1365-3040.2007.01642.x.

Qiu, H., Smolders, E., 2017. Nanospecific phytotoxicity of CuO nanoparticles in soils disappeared when bioavailability factors were considered. Environ. Sci. Technol. 51 (20), 11976-11985. https://doi.org/10.1021/acs.est.7b01892.

Rai, M., Lngle, A.P. Pandit, R., Paralikar, P., Shende, S., Gupta, I., Biswas J.K., da Silva, S.S., 2018. Copper and copper nanoparticles: role in management of insect-pests and pathogenic microbes. Nanotechnol. Rev. 7 (4), 303-315. https://doi.org/10.1515/ntrev-2018-0031.

Rajput, V.D., Minkina, T., Sushkova, S., Mandzhieva, S., Fedorenko, A., Lysenko, V., Bederska-Błaszczyk, M., Olchowik, J., Tsitsuashvili, V., Chaplygin, V., 2019. Structural and ultrastructural changes in nanoparticle exposed plants. Nanoscience for Sustainable Agriculture. Springer, Cham. 281-295. https://doi.org/10.1007/978-3-319-97852-9_13.

Raliya, R., Saharan, V., Dimkpa, C., Biswas, P., 2017. Nanofertilizer for precision and sustainable agriculture: current state and future perspectives. J. Agric. Food. Chem. 66 (26), 6487-6503. https://doi.org/10.1021/acs.jafc.7b02178.

Rawat, S., Pullagurala, V.L.R., Hernandez-Molina, M., Sun, Y., Niu, G., Hernandez-Viezcas, J., Peralta-Videa J.R., Gardea-Torresdey, J., 2017. Impacts of copper oxide nanoparticles in bell pepper (*Capsicum annum* L.) plants: A full life cycle study. Environ. Sci. Nano. 5, 83-95. https://doi: 10.1039/C7EN00697G.

Ross, K.A., Brenza, T.M., Binnebose, A.M., Phanse, Y., Kanthasamy, A.G., Gendelman, H.E., Salem, A.K., Bartholomay, L.C., Bellaire, B.H., Narasimhan, B., 2015. Nano-enabled delivery of diverse payloads across complex biological barriers. J. Control. Release. 219, 548-559. https://doi.org/10.1016/j.jconrel.2015.08.039.

Sakai, T., Kogiso, M., 2008. Soy isoflavones and immunity. J. Med. Invest. 55, 167-173. https://doi.org/10.2152/jmi.55.167.

Sekine, R., Marzouk, E.R., Khaksar, M., Scheckel, K.G., Stegemeier, J.P., Lowry, G.V., Donner, E., Lombi, E., 2017. Aging of dissolved copper and copper-based nanoparticles in five different soils: short-term kinetics vs. long-term fate. J. Env. Qual. 46 (6), 1198-1205. https://doi:10.2134/jeq2016.12.0485.

Servin, A., Elmer, W., Mukherjee, A., Torre-Roche, R.D., Hamdi, H., White, J.C., Bindraban, P., Dimkpa, C., 2015. A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. J. Nanopart. Res. 17, 92. https://doi.org/10.1007/s11051-015-2907-7.

Shah, V., Belozerova, I., 2009. Influence of metal nanoparticles on the soil microbial community and germination of lettuce seeds. Water Air Soil Pollut. 197, 143-148. https://doi.org/10.1007/s11270-008-9797-6.

Shaw, A.K., Ghosh, S., Kalaji, H.M., Bosa, K., Brestic, M., Zivcak, M., Hossain, Z., 2014. Nano-CuO stress induced modulation of antioxidative defenseand photosynthetic performance of Syrianbarley (*Hordeum vulgare* L.). Environ. Exp. Bot. 102, 37-47. https://doi.org/10.1016/j.envexpbot.2014.02.016.

Shaw, A.K., Hossain, Z., 2013. Impact of nano-CuO stress on rice (*Oryza sativa* L.) seedlings. Chemosphere. 93 (6), 906-915. https://doi.org/10.1016/j.chemosphere.2013.05.044.

Shi, J., Abid, A.D., Kennedy, I.M., Hristova, K.R., Silk, W.K., 2011. To duckweeds (*Landoltia punctata*), nanoparticulate copper oxide is more inhibitory than the soluble copper in the bulk solution. Environ. Pollut. 159 (5), 1277-1282. https://doi.org/10.1016/j.envpol.2011.01.028.

Shi, J.Y., Peng, C., Yang, Y.Q., Yang, J.J., Zhang, H., Yuan, X.F., Chen, Y.X., Hu, T.D., 2014. Phytotoxicity and accumulation of copper oxide nanoparticles to the Cu-tolerant plant *Elsholtzia splendens*. Nanotoxicol. 8 (2), 179-188. https://doi.org/10.3109/17435390.2013.766768.

Silva, T.U., Pokhrel, L.R., Dubey, B., Tolaymat, T.M., Maier, K.J., Liu, X., 2014. Particle size, surface charge and concentration dependent ecotoxicity of three organo-coated silver nanoparticles: general linear model-predicted and observed toxicity. Sci. Total Environ. 468-469, 968-976. https://doi.org/10.1016/j.scitotenv.2013.09.006.

Singh, R.J., Nelson, R.L., Chung, G., 2007. Genetic resources, chromosome engineering, and crop improvement, chapter 2: soybean (*Glycine max* (L.) Merr.). CRC press, Taylor & Francis Group. pp, 14-49.

Stampoulis, D., Sinha, S.K., White, J.C., 2009. Assay-dependent phytotoxicity of nanoparticles to plants. Environ. Sci. Technol. 43 (24), 9473-9479. https://doi.org/10.1021/es901695c.

The world's healthiest foods. July 24, 2020. Available at: http://www.whfoods.com/genpage.php?tname=foodspice&dbid=58.

Trujillo-Reyes, J., Majumdar, S., Botez, C.E., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2014. Exposure studies of core-shell $Fe/Fe_3O_4$ and Cu/CuO NPs to lettuce (*Lactuca sativa*) plants: are they a potential physiological and nutritional hazard? J. Hazard. Mater. 267, 263-455. http://dx.doi.org/10.1016/j.jhazmat.2013.11.067.

U.S. Department of Agriculture (USDA), Agricultural Research Service's Food Data Central, 2019. Available at: https://fdc.nal.usda.gov/.

U.S. Food and Drug Administration, 2013. Guidance for Industry: a food labeling guide (14. appendix f: calculate the percent daily value for the appropriate nutrients).

U.S. Food and Drug Administration, 2017. Food labeling: revision of the nutrition and supplement facts labels and serving sizes of foods that can reasonably be consumed at one eating occasion; dual-column labeling; updating, modifying, and establishing certain reference amounts customarily consumed; serving size for breath mints; and technical amendments; proposed extension of compliance dates.

UN (United Nations Department of Economic and Social Affairs, Population Division), 2019. World Population Prospects: the 2019 Revision.

US Food and Drug Administration (FDA). Food Labeling: Revision of the Nutrition and Supplement Facts Labels. Federal Register, Vol. 81, No. 103, Friday, May 27, 2016. Docket No. FDA–2012–N–1210; RIN 0910–AF22.

Wang, Z., Xie, X., Zhao, J., Liu, X., Feng, W., White, J.C., Xing, B., 2012. Xylem-and phloem-based transport of CuO nanoparticles in maize (*Zea mays* L.). Environ. Sci. Technol. 46 (8), 4434-4441. https://doi.org/10.1021/es204212z.

Welch, R., Graham, R., 2004. Breeding for micronutrients in staple food crops from a human nutrition perspective. J. Exp. Bot. 55 (396), 353-364. https://doi.org/10.1093/jxb/erh064.

Xu, R., Hu, W., Zhou, Y., Zhang, X., Xu, S., Guo, Q., Qi, P., Chen, L., Yang, X., Zhang, F., Liu, L., Qiu, L., Wang, J., 2020. Use of near-infrared spectroscopy for the rapid evaluation of soybean [*Glycine max* (L.) Merri.] water soluble protein content. Spectrochim. Acta A: Molecular and Biomolecular Spectroscopy. 224, 117400. https://doi.org/10.1016/j.saa.2019.117400.

Yang, Z.Z., Chen, J., Dou, R.Z., Gao, X., Mao, C.B., Wang, L., 2015. Assessment of the phytotoxicity of metal oxide nanoparticles on two crop plants, maize (*Zea mays* L.) and rice (*Oryza sativa* L.). Int. J. Environ. Res. Public Health. 12 (12), 15100-15109. https://doi.org/10.3390/ijerph121214963.

Yuan, M., Wang, S., Chu, Z., Li, X., Xu, C., 2010. The bacterial pathogen Xanthomonas oryzae overcomes rice defenses by regulating host copper redistribution. Plant Cell. 22, 3164-3176. https://doi.org/10.1105/tpc.110.078022.

Yusefi-Tanha, E., Fallah, S., Rostamnejadi, A., Pokhrel, L.R., 2020. Particle size and concentration dependent toxicity of copper oxide nanoparticles (CuONPs) on seed yield and antioxidant defense system in soil grown soybean (*Glycine max* cv. Kowsar). Sci. Total Environ. 715, 136994. https://doi.org/10.1016/j.scitotenv.2020.136994.

Zhou, W., Liu, Y.L., Stallworth, A.M., Ye, C., Lenhart, J.J., 2016. Effects of pH, electrolyte, humic acid, and light exposure on the long-term fate of silver nanoparticles. Environ. Sci. Technol. 50 (22), 12214-12224. https://doi.org/10.1021/acs.est.6b03237.

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**FIGURES & TABLES**

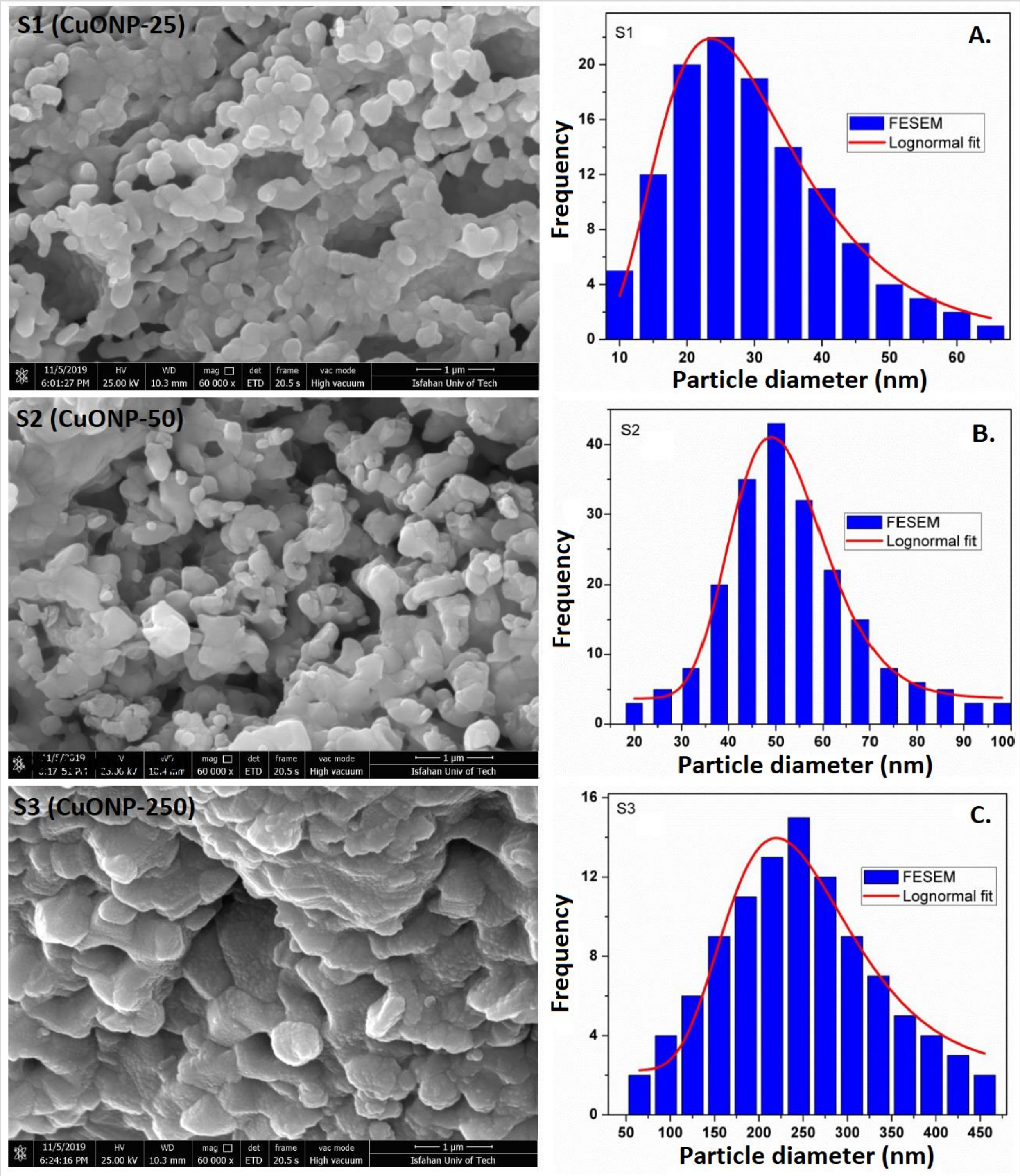


**Fig. 1.** FE-SEM micrographs of CuONPs samples with the fitted particle size distribution (PSD) to a log-normal function: (**A**) S1 with mean diameter of 25 nm, labeled as CuONP-25; (**B**) S2 with mean diameter of 50 nm, labeled as CuONP-50; and (**C**) S3 with mean diameter of 250 nm, labeled as CuONP-250.

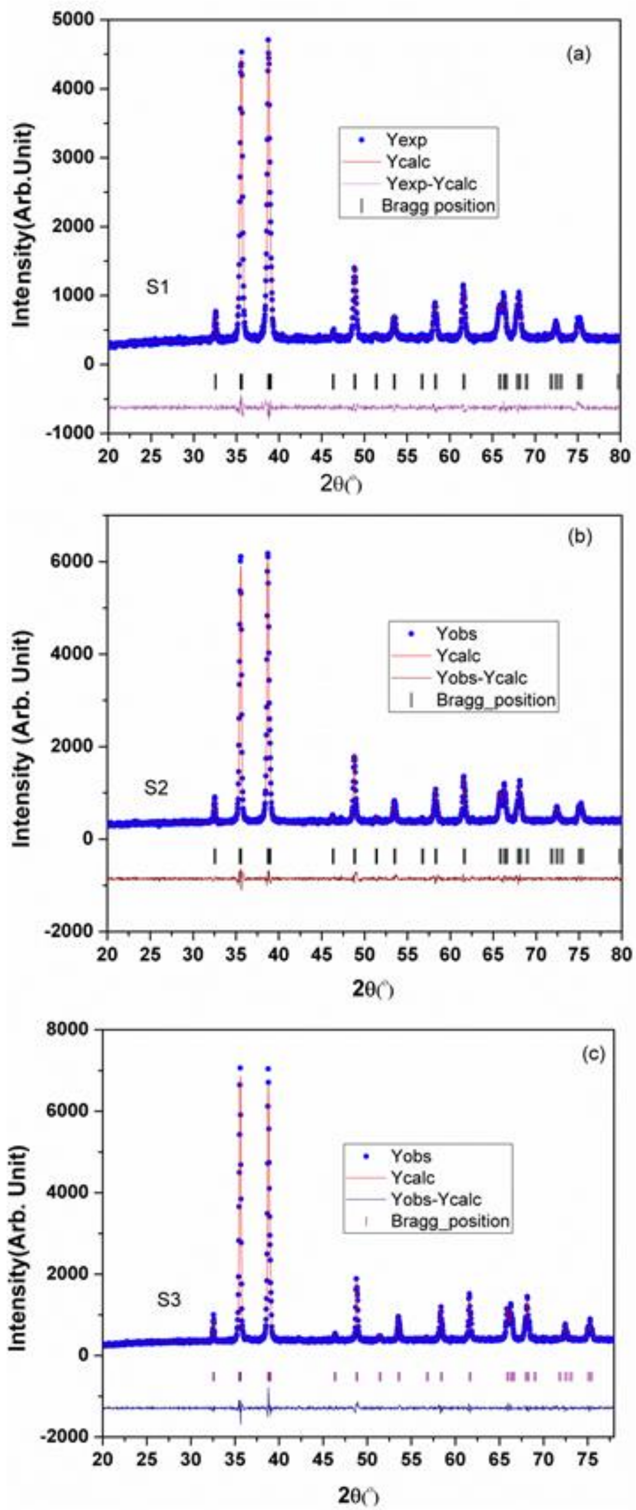


**Fig. 2.** The Reitveld analysis of the XRD pattern of the CuONPs: (a) S1, (b) S2 and (c) S3. (a) S1 with mean diameter of 25 nm; (b) S2 with mean diameter of 50 nm; and (c) S3 with mean diameter of 250 nm.

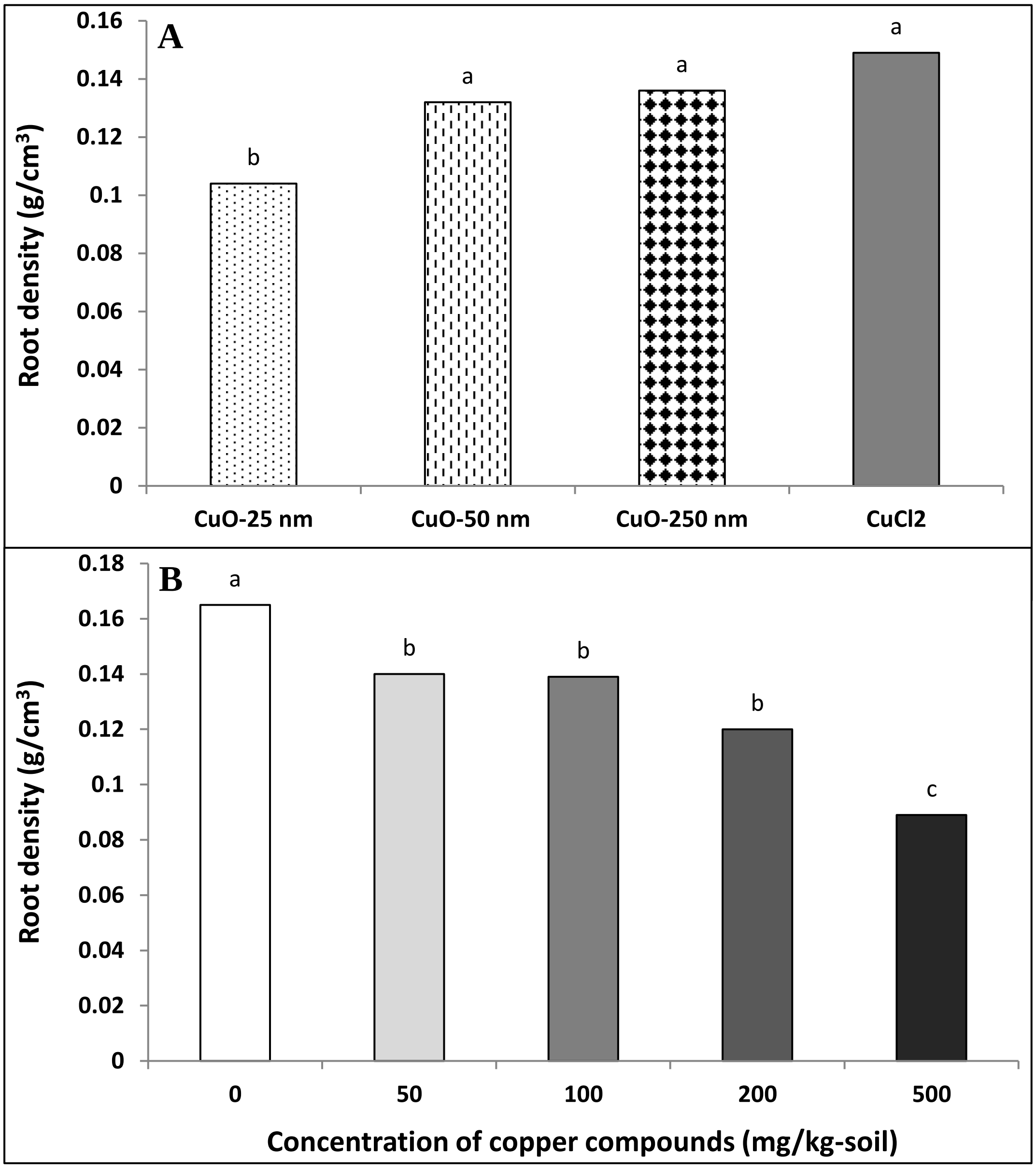


**Fig. 3.** Effect of copper compounds types (A) and concentration of copper compounds (B) on soybean root density. Same letter above the bars indicates not significant difference according to the LSD test ($p \leq 0.05$).

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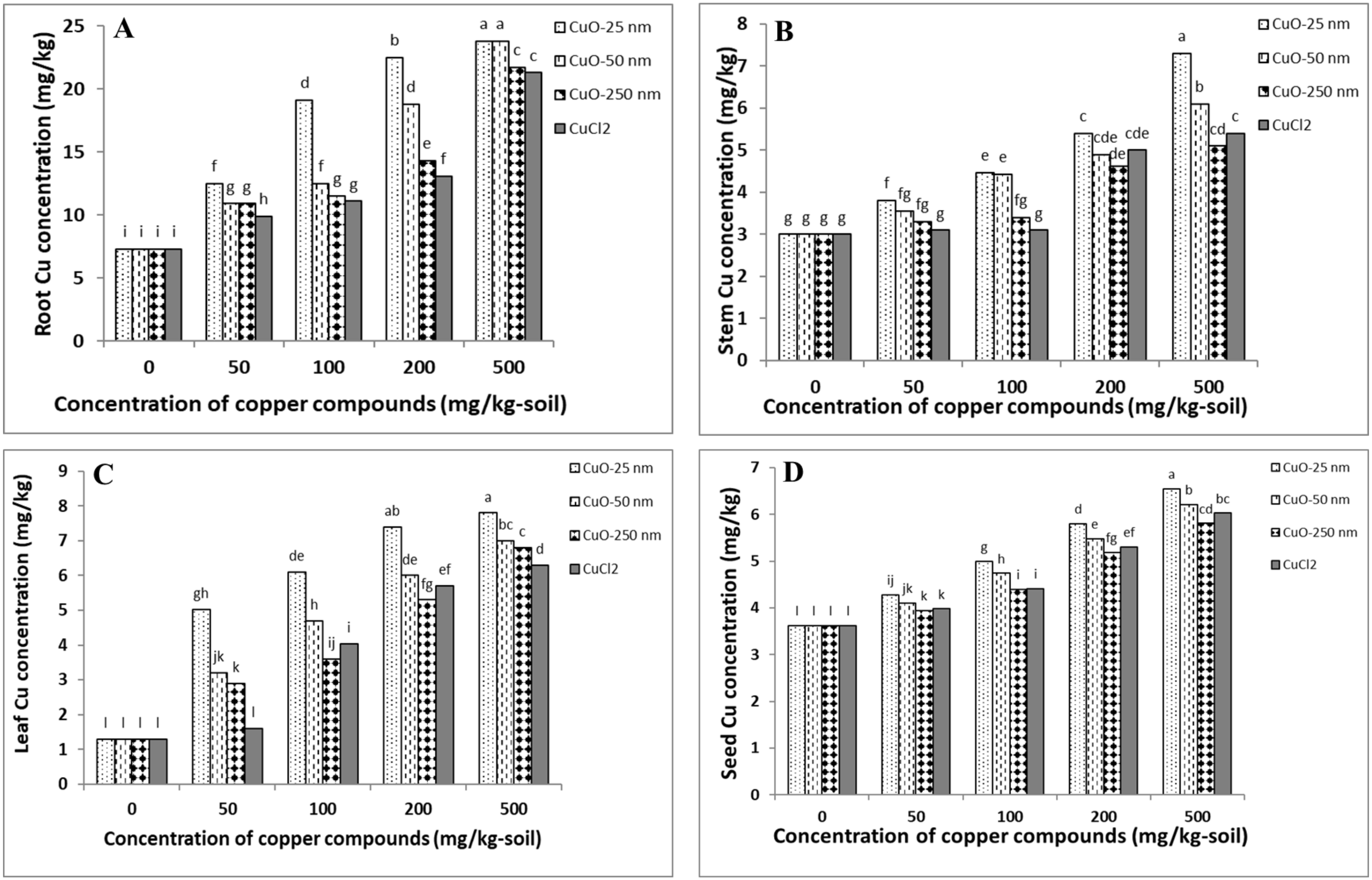


**Fig. 4.** Cu uptake in soybean root (A), stem (b), leaf (C), and seed (D) upon exposure to soil amended with CuONP-25 nm, CuONP-50 nm, CuONP-250 nm, and $CuCl_2$, at different concentrations. Same letter above the bars indicates not significant difference according to the LSD test ($p \leq 0.05$).

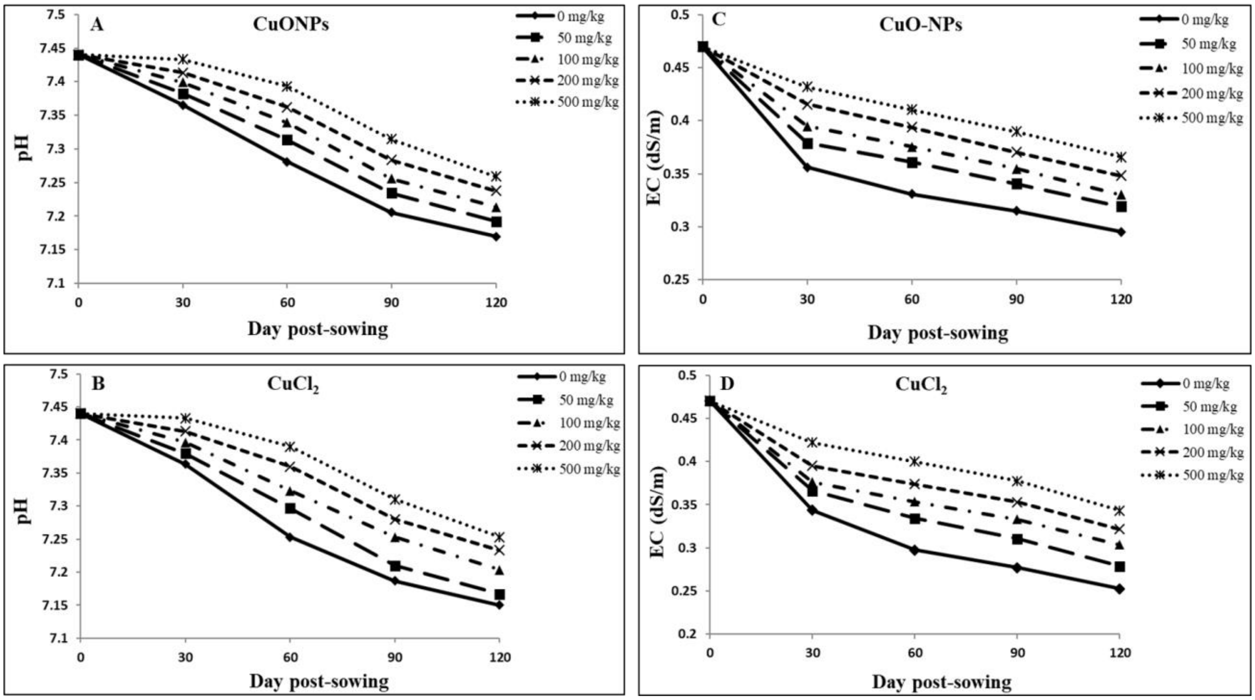


**Fig. 5.** Changes in soil pH (A, B) and electrical conductivity (EC) (C, D) of soil amended with CuONPs (A, C) and $CuCl_2$ (B, D) during the full growth period of 120 days in soybean. The data are averaged for all three types of CuONPs as they were similar among the NPs.

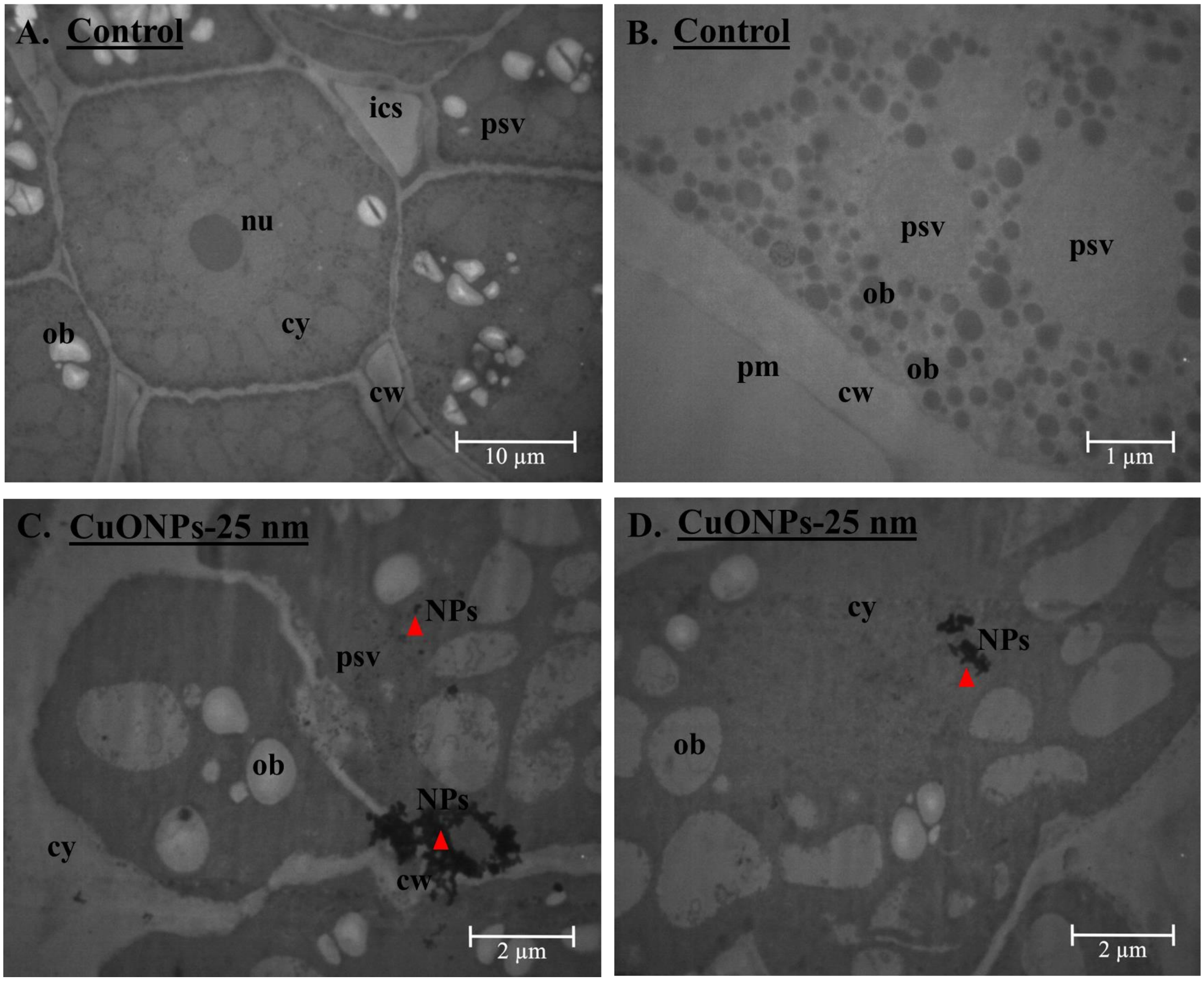


**Fig. 6.** TEM analysis of ultrastructure of soybean seed embryo with CuONPs-25 nm treatment at 500 mg/kg-soil (C, D) and compared with control seeds (no nanoparticles) (A, B). Electron dense metal aggregates are clearly visible within cell wall (cw)/ plasma membrane (pm) including within the cytoplasm (cy) and/or protein storage vacuoles (psv) for the seeds with CuONPs-25 nm treatment at 500 mg/kg-soil (C, D). nu = nucleus, ics = intracellular space, ob = oil bodies, NPs = nanoparticles (red triangle).

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**TABLES:**

**Table 1. Summary of the structural parameters of CuONPs obtained from the Rietveld analysis of the XRD patterns.**

| Sample | S1 | S2 | S3 |
|---|---|---|---|
| Structure | Monoclinic | Monoclinic | Monoclinic |
| Space group | *C2/c* | *C2/c* | *C2/c* |
| Lattice parameters | | | |
| *a* (Å) | 4.68369 | 4.68678 | 4.68469 |
| *b* (Å) | 3.42223 | 3.42659 | 3.42223 |
| *c* (Å) | 5.12774 | 5.13265 | 5.12964 |
| α (°) | 90.00 | 90.00 | 90.00 |
| β (°) | 99.37073 | 99.40 | 99.49814 |
| γ (°) | 90.00 | 90.00 | 90.00 |
| *Unit cell Volume* $(Å)^3$ | 81.094 | 81.321 | 81.111 |
| *particle size* (nm) | 25 | 50 | 250 |
| *Reliability factors* | | | |
| *Weighted profile factor* ($R_{wp}$) | 13.3 | 12.1 | 12.9 |
| *Profile factor* ($R_p$) | 16.1 | 14.7 | 17.4 |
| *Expected R-factor* ($R_{exp}$) | 10.41 | 9.78 | 10.87 |
| *Bragg R-factor* ($R_{Bragg}$) | 1.65 | 1.29 | 1.10 |
| *R-factor* ($R_F$) | 1.16 | 1.01 | 0.843 |
| *Chi squared* ($\chi^2$) | 1.85 | 1.80 | 1.90 |

**Table 2. Analysis of variance (df, *P* value) for select root morphological parameters of soybean grown in soil treated with different concentration of copper compounds types.**

| Source of variation | | Root dry weight | Root length | Root volume | Root area | Root density |
|---|---|---|---|---|---|---|
| Copper compounds types ($Cu_{type}$) | df | 3 | 3 | 3 | 3 | 3 |
| | *P* | <.0001 | <.0001 | <.0001 | <.0001 | 0.0001 |
| Compounds concentration (C) | df | 4 | 4 | 4 | 4 | 4 |
| | *p* | <.0001 | <.0001 | <.0001 | <.0001 | <.0001 |
| $Cu_{type} \times C$ | df | 12 | 12 | 12 | 12 | 12 |
| | *p* | 0.0188 | 0.0028 | 0.0572 | 0.0009 | 0.5139 |

**Table 3. Effect of CuONPs and $CuCl_2$ on root dry weight and root length of soybean. Means with a similar letter are not significant difference, according to LSD test ($p \leq 0.05$).**

| Concentration (mg/kg) | Root dry weight (g/plant) | | | | Root length (cm) | | | |
|---|---|---|---|---|---|---|---|---|
| | CuO-NPs (nm) | | | $CuCl_2$ | CuO-NPs (nm) | | | $CuCl_2$ |
| | 25 | 50 | 250 | | 25 | 50 | 250 | |
| **0** | 5.45[a] | 5.45[a] | 5.45[a] | 5.45[a] | 38.60[a] | 38.60[a] | 38.60[a] | 38.60[a] |
| **50** | 3.02[f] | 4.08[bc] | 4.42[bc] | 4.81[ab] | 34.22[bc] | 34.22[bc] | 35.16[abc] | 37.83[a] |
| **100** | 2.20[ghi] | 3.27[def] | 4.02[bcd] | 4.30[bc] | 26.50[ef] | 32.53[cd] | 34.01[bc] | 37[ab] |
| **200** | 1.53[ij] | 2.63[fgh] | 2.99[fg] | 3.83[cde] | 22.50[g] | 26.50[ef] | 29.83[de] | 32.33[cd] |
| **500** | 0.94[j] | 1.67[ij] | 2.05[hi] | 3.16[ef] | 18.83[h] | 25.03[fg] | 26.05[f] | 30[d] |

**Table 4. R-squared values based on the linear regression lines for multiple parameters tested for different sized CuONPs and $CuCl_2$ treatments in soybean. If the co-efficient of determination (R-squared) value for the linear regression line is 65% or higher the concentration-response curves were deemed linear.**

| Parameters | R-squared | | | |
|---|---|---|---|---|
| | CuONP-25 nm | CuONP-50 nm | CuONP-250 nm | $CuCl_2$ |
| **Root dry weight** | 0.60 (NL) | 0.78 (L) | 0.87 (L) | 0.85 (L) |
| **Root length** | 0.75 (L) | 0.76 (L) | 0.89 (L) | 0.88 (L) |
| **Root volume** | 0.68 (L) | 0.65 (L) | 0.43 (NL) | 0.67 (L) |
| **Root area** | 0.72(L) | 0.71 (L) | 0.72 (L) | 0.84 (L) |
| **Root Cu uptake** | 0.64 (NL) | 0.90 (L) | 0.97 (L) | 0.99 (L) |
| **Stem Cu uptake** | 0.96 (L) | 0.90 (L) | 0.85 (L) | 0.79 (L) |
| **Leaf Cu uptake** | 0.55 (NL) | 0.74 (L) | 0.87 (L) | 0.72 (L) |
| **Seed Cu uptake** | 0.85 (L) | 0.87 (L) | 0.89 (L) | 0.90 (L) |

**"L" denotes linear, and "NL" denotes nonlinear concentration-response relationships.**

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**Table 5. Effect of CuONPs and $CuCl_2$ on root volume and root area of soybean. Means with a similar letter are not significant difference, according to LSD test ($p \leq 0.05$).**

| Concentration (mg/kg) | Root volume ($cm^3$) | | | | Root area ($cm^2$) | | | |
|---|---|---|---|---|---|---|---|---|
| | CuO-NPs | | | $CuCl_2$ | CuO-NPs | | | $CuCl_2$ |
| | 25 | 50 | 250 | | 25 | 50 | 250 | |
| **0** | $33.34^{a}$ | $33.34^{a}$ | $33.34^{a}$ | $33.34^{a}$ | $126.94^{a}$ | $126.94^{a}$ | $126.94^{a}$ | $126.94^{a}$ |
| **50** | $28.16^{bc}$ | $28.66^{bc}$ | $28.83^{bc}$ | $30.83^{ab}$ | $110.01^{c}$ | $110.89^{bc}$ | $112.84^{bc}$ | $121^{ab}$ |
| **100** | $22.66^{efg}$ | $23.33^{defg}$ | $25.16^{cdef}$ | $27.50^{bcd}$ | $86.73^{fgh}$ | $97.44^{de}$ | $103.45^{cd}$ | $112.98^{bc}$ |
| **200** | $17^{hi}$ | $21^{fgh}$ | $25^{cdef}$ | $27.16^{bcd}$ | $69.27^{ij}$ | $83.60^{gh}$ | $96.70^{def}$ | $104.95^{cd}$ |
| **500** | $16^{i}$ | $19.33^{ghi}$ | $24.83^{cdef}$ | $25.48^{cde}$ | $61.21^{j}$ | $77.96^{hi}$ | $90.13^{efg}$ | $97.86^{de}$ |

**Table 6. Analysis of variance (df, *p* value) for copper (Cu) compound types, concentrations, and their interaction term for Cu uptake in different tissues (root, stem, leaf, and seed) in soybean grown in soil treated with different concentrations of Cu compound types.**

| Source of variation | | Copper concentration | | | |
|---|---|---|---|---|---|
| | | Root | Stem | Leaf | Seed |
| Cu compounds types ($Cu_{type}$) | df | 3 | 3 | 3 | 3 |
| | *P* | <.0001 | <.0001 | <.0001 | <.0001 |
| Compounds concentration (C) | df | 4 | 4 | 4 | 4 |
| | *p* | <.0001 | <.0001 | <.0001 | <.0001 |
| $Cu_{type} \times C$ | df | 12 | 12 | 12 | 12 |
| | *p* | <.0001 | 0.0005 | <.0001 | 0.0083 |

**Table 7. Cu concentrations in soybean seeds compared to Recommended Daily Allowances (RDA), Cu concentrations in chickpeas seeds, and % Daily Values (DV) based on US FDA recommendations for adults and children aged 4 years and older (USFDA, 2016).**

| Experimental | | | | Cu concentration in Chickpeas seeds (ug Cu/100 g seed) | Fold higher than Chickpeas seed Cu concentration (per serving of ½ cup) | Recommended Dietary Allowances (RDA) for Cu intake for adults and children aged 4 years and older (ug) | % Daily Values (DV)* | World's Healthiest Foods Rating[#] | Food items that provide lower Cu than our soybean seeds[£] |
|---|---|---|---|---|---|---|---|---|---|
| Treatment types | Concentration applied (mg Cu/kg soil) | mg Cu/kg Soybean seed | ug Cu/100 g Soybean seed | | | | | | |
| **CuONPs-25 nm** | 50 | 4.28 | 428 | 289 | x1.48 | 900 | 47.5 | Good | Atlantic Salmon (wild, cooked), Avocado, Asparagus, Cream of Wheat, Dried Figs, Ground Turkey, Greek Yogurt, Non-fat Milk, Pasta, Sesame seeds, Whole Wheat |
| | 100 | 5.00 | 500 | | x1.73 | | 55.5 | Very Good | |
| | 200 | 5.80 | 580 | | x2.00 | | 64.4 | Very Good | |
| | 500 | 6.55 | 655 | | x2.30 | | 73.0 | Very Good | |
| **CuONPs-50 nm** | 50 | 4.11 | 411 | | x1.42 | | 45.6 | Good | |
| | 100 | 4.74 | 474 | | x1.64 | | 53.0 | Very Good | |
| | 200 | 5.48 | 548 | | x1.90 | | 61.0 | Very Good | |
| | 500 | 6.21 | 621 | | x2.15 | | 69.0 | Very Good | |
| **CuONPs-250 nm** | 50 | 3.94 | 394 | | x1.36 | | 44.0 | Good | |
| | 100 | 4.4 | 440 | | x1.52 | | 49.0 | Good | |
| | 200 | 5.19 | 519 | | x1.80 | | 58.0 | Very Good | |
| | 500 | 5.81 | 581 | | x2.01 | | 65.0 | Very Good | |
| **$CuCl_2$** | 50 | 3.98 | 398 | | x1.38 | | 44.2 | Good | |
| | 100 | 4.41 | 441 | | x1.53 | | 49.0 | Good | |
| | 200 | 5.31 | 531 | | x1.84 | | 59.0 | Very Good | |
| | 500 | 6.03 | 603 | | x2.10 | | 67.0 | Very Good | |

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|---|---|---|---|---|---|---|---|---|---|
| **Control** | 0 | 3.62 | 362 | | x1.25 | | 40.2 | Good | |

**100 g of soybean seed is assumed to be equivalent to 100 g of chickpeas seeds per serving of ½ cup, which is equivalent to 3.5 ounces. % DV = % RDA.**
***The DV for copper on the new Nutrition Facts and Supplement Facts labels and used for the values in Table 4 is 0.9 mg (900 ug) for adults and children aged 4 years and older (US FDA, 2016). Foods providing 20% or more of the DV are considered to be high sources of a nutrient.**
**[#]The "World's Healthiest Foods" Rating is based on a simple rule: Excellent, if DV ≥75%; Very Good, if DV ≥ 50%; Good, if DV ≥ 25% (http://www.whfoods.com/genpage.php?tname=foodspice&dbid=58).**
**[£]Food items for which Cu levels are compared are adopted from the U.S. Department of Agriculture, Agricultural Research Service's FoodData Central (USDA, 2019).**