**Modulation of CuO nanoparticles toxicity to green pea (*Pisum sativum* Fabaceae) by the
phytohormone indole-3-acetic acid**

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Abstract

The response of plants to copper oxide nanoparticles (nano-CuO) in presence of exogenous phytohormones is unknown. In this study, green pea (*Pisum sativum*) plants were cultivated to full maturity in soil amended with nano-CuO (10-100 nm, 74.3% Cu), bulk-CuO (bCuO, 100-10,000 nm, 79.7% Cu), and CuCl₂ at 50 and 100 mg/kg and indole-3-acetic acid (IAA) at 10 and 100 µM. Results showed that IAA at 10 and 100 µM, averaged over all Cu treatments, reduced the number of plants by ~23% and ~34%, respectively. IAA at 10 µM, nano-CuO at 50 mg/kg, b-CuO at 50 mg/kg, and CuCl₂ at 100 mg/kg reduced pod biomass by about 50%. Although some combinations of IAA, mainly at 100 µM, with the Cu compounds altered nutrient accumulation in tissues, none of them affected pod elements. Conversely, without IAA, nano-CuO at 50 mg/kg, increased pod Fe and Ni by 258% and 325%, respectively, while bCuO at 100 mg/kg increased pod Ni by 275%, compared with control. With IAA at 10 µM, nano-CuO (100 mg/kg) and bCuO (50 mg/kg) increased stem Cu by ~84% and ~78%. When IAA increased to 100 µM, nano-CuO and bCuO reduced stem Ca by 32 and 37%, and Mg by ~35%. Results suggest that both the nano-CuO and bCuO could improve the nutritional quality of pea pods, while exogenous IAA combined with Cu-based compounds could impact green pea production since these treatments reduced the number of plants and pod biomass.

Keywords: Copper oxide nanoparticles, green pea, auxins, biomass, nutrient elements, phytohormones

Introduction

Copper-based nanoparticles (NPs) are profusely used due to their optical, thermal, electrical, antibacterial, and catalytic applications (Peralta-Videa et al. 2016). The global production of Cu/CuO nanoparticles (NPs) for 2010 was estimated in 200 tons, 36 of which ended up in soil and 11 in bodies of water (Keller et al. 2013). Very recently, nanoparticulate forms of copper are starting to be used in agriculture for specific purposes (Servin et al., 2015). For instance, CuO, Cu₂O NPs and Cu/Cu₂O nanocomposite have been used as alternative to non-nano Cu products to combat fungal diseases in tomato (Servin et al., 2015). This suggests that the global production of Cu-based nanoforms would increase to unanticipated levels. However, little is known about their physiological and biochemical effects in agricultural crops (Du et al., 2017) and very few studies have covered the full life cycle of the plants (Zuverza-Mena et al. 2017). From previous publications, it is known that nano-CuO, at different concentrations, alters plant growth and development by increasing the reactive oxygen species (ROS) production and unbalancing homeostasis of essential elements (Du et al., 2017). Dimkpa et al. (2012) reported that these NPs, at 500 mg Cu/kg, decreased shoot growth, chlorophyll content, and oxidized glutathione in wheat seedling (*Triticum aestivum*) roots. Zhenyu et al. (2012) exposed corn (*Zea mays*) seedlings to 100 mg/L nano-CuO in hydroponics and found a reduction in root length and leaf surface area. In hydroponically grown lettuce (*Lactuca sativa*), nano-CuO at 20 mg/L reduced water content, root length and dry biomass (Trujillo-Reyes et al., 2014). In soybeans (*Glycine max*) nano-CuO reduced shoot growth, plant weight, and chlorophyll content (Nair and Chung, 2014). Additionally, Hong et al. (2015) reported that nano-CuO reduced root length in hydroponically grown alfalfa (*Medicago sativa*) and lettuce.

Plants respond to external stimuli through different types of endogenous hormones (Gaspar et al., 1996; Barbafieri et al., 2012). One of the most studied phytohormones are auxins, more specifically, indole-3-acetic acid (IAA). At the correct amount, IAA promotes stem elongation, tropism, adventitious root formation, and cell division, among others (López et al., 2007b; Barbafieri et al., 2012). Samish and Gur (1962) reported that IAA at 50 mg/L improved bud-take in avocado, while at 200 mg/L increase bud abscission. Zhao (2010), mentioned that an increment in IAA may modify plant development. Because of that, IAA has been used in agriculture to enhance crop growth and rooting in cuttings (Barbafieri et al. 2012). In a previous study, López et al. (2007a), showed that IAA significantly increased lead (Pb) uptake and translocation in hydroponically grown alfalfa, exposed to Pb plus ethylenediaminetetraacetic acid (EDTA). Piotrowska-Niczyporuk et al. (2012) reported that the unicellular green algae *Chorella vulgaris*, exposed to heavy metals and several phytohormones increased its carotenoids, chlorophyll, protein, and glutathione contents, as well as superoxide dismutase, ascorbate, and catalase enzymatic activities. This suggests exogenous phytohormones can mitigate stress symptoms and the phytotoxicity of heavy metals (Dimkpa et al., 2008; Choudhary et al., 2010; Piotrowska-Niczyporuk et al., 2012).

A recent study has shown that agricultural fields, amended with biosolids from wastewater treatment plants, contain nanomaterials used in consumer products (Yang et al., 2014). Green pea (*Pisum sativum* L.), a legume rich in protein, aminoacids, vitamins, and minerals (Iqbal et al. 2006) could, thus, be unintentionally exposed to NPs when grown in biosolid amended soils. Moreover, bacteria isolated from pea plant root nodules have shown to produce IAA (Tariq et al. 2014). This suggests that green pea plants associated with high IAA producing bacteria could be exposed to an unusual interaction of IAA and nano-CuO, with unknown consequences.

The objectives of this research were to determine the effects of nano-CuO and IAA on the agronomical, physiological, and biochemical traits of pea plants. Spectroscopic and biochemical techniques were used to evaluate the effects of treatments through the life cycle of the plants.

Materials and methods

Characteristics of nCuO and bCuO and suspensions/solutions preparation

Copper oxide NPs (nano-CuO), bulk CuO (bCuO), and copper chloride ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$) (Sigma-Aldrich), were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). According to previous characterization (Hong et al. 2015), nano-CuO have a primary particle size of 10-100 nm, Cu content of 74.3 (wt. %), hydrodynamic diameter of 280 ± 15 (nm), and a ζ - potential of -34.4 ± 0.5 (mV). The bCuO has a primary particle size of 100-10,000 (nm), Cu content of 79.7 (wt. %), hydrodynamic diameter of 376 ± 26 (nm), and a ζ - potential of $-42.7 \pm 0.153 \pm$ (mV). Suspensions of nano-CuO, bCuO, and CuCl_2 were prepared at 0, 50, and 100 mg/kg (in terms of Cu content) in 250 mL volumetric flasks, using Millipore water. To avoid aggregation, suspensions/solutions were sonicated for 30 min in a water bath at 25 °C (Crest Ultrasonics, Trenton, NJ Model 275 DA; 120 volt, 3 amp, 59/60 Hz). Levels of CuO were selected after Nair and Chung (2014), with a reduced number of concentrations, due to the number of Cu compounds. Solutions of IAA (Aldrich Chemicals) were prepared at 0, 10, and 100 μM , as described by Lopez et al. (2007a). Table S1 (Supplementary Material) shows the combination of products to generate treatments used in this study.

Soil collection and application of the suspensions

Natural soil was collected from Socorro, TX (N 31° 40.489', W 106° 17.198', elevation: 1,115 m asl). To avoid root debris and residues of fertilizers, the top portion (first ~15 cm) was removed

and the samples were taken from a depth of 15-60 cm (http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs144p2_051273.pdf). The soil was air-dried and sieved through a 6 mm mesh prior to experimental use. The soil had 19 % clay, 44% silt, and 36% sand, and was classified as loam (one of the best agricultural soils, according to USDA soil texture classification). Other properties include a pH of 7.8 ± 0.021 , electrical conductivity (EC) of $1705 \pm 47.6 \mu\text{S}/\text{cm}$, and total dissolved solids (TDS) of $847.5 \pm 23.8 \text{ mg}/\text{L}$ (Hanna Instruments HI 9813-6 Portable pH/EC/TDS/Temperature Meter). The elemental analysis of the soil is shown in Table S2 (SM).

A total of 84 general purpose plastic pots were previously washed with a mild solution of 5% nitric acid (HNO_3) and deionized water to remove metal contaminants. Pots were filled with 2 kg of natural soil amended with the Cu suspensions/solutions, manually mixed until getting a homogenous mixture, and kept for 24 h for stabilization. Four replicate/treatment including control (no NPs, no IAA) were established.

Seed planting and growth conditions

Green pea seeds (Little Marvel Dwarf 1454, Ferry Morse, Norton, MA) were soaked in a 2% sodium hypochlorite solution for five min, rinsed five times with deionized water, and immersed in Millipore water for 24 h to hydrate. Each pot was seeded with five seeds (~2.5 cm deep) and placed in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) set at 14 h photoperiod, 25/20 °C day/night, 65-70 relative humidity and light intensity of $340 \mu\text{mole m}^{-2}\text{s}^{-1}$. Five days after planting, the pots were added with the respective IAA solution. To avoid root rot, in the subsequent five days, the fungicide (OHP® CHIPCO® 26019 N/G) was applied as a drench, following recommendations of the manufacturer. All plants were watered daily with 50-60 mL of deionized water. No additional fertilizer or nutrient solution was added.

Treatment effects on physiological/agronomical parameters

At 45 days (beginning of blossom, maximum photosynthates accumulation) and 90 days (full maturity), sets of plants (three replicate/treatment) were harvested to evaluate physiological/agronomical traits such as plant growth, dry biomass, chlorophyll, carotenoids, seed production, and nutrient elements, among others. Plants were severed in leaves, stems, and roots, washed with 0.01 M HNO₃ solution, and rinsed three times with Millipore water to remove external contaminants. All plants were counted to record the plant survival at 45 days. Roots were measured from the main apex to the crown and stems from the crown to the main shoot apex. Samples were placed in paper envelopes and oven dried at 60 °C for 72 h for further analysis. Similar procedure was followed for the 90-day old plants.

Determination of Cu and other nutritional elements in dry plant tissues

Dried samples of plant organs were digested with 4 mL of HNO₃ (67-70%) and placed in a DigiPREP MS digestion block (SCP Science, NY) for 45 min at 115 °C. The digests were diluted with Millipore water up to 50 ml and analyzed for B, Cu, Fe, Mo, Mn, Ni, Zn, Ca, K, Mg, P, and S concentrations by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Perkin-Elmer optima 4300 DV). *Aqua-regia* digested soil samples were analyzed for the same elements determined in plants. For quality assurance/quality control, blanks, spikes, and certified standard reference material for plant (spinach leaves NIST-SRM 1570a) and soil (NIST-SRM 2709a San Joaquin Soil) were analyzed, obtaining 98% of recovery for Cu, with a detection limit of 1.8 µg/L. In addition, 0.5 mg/L of a multi-element standard solution was analyzed every 10 samples to monitor the matrix effect on the analytes and for quality assurance/quality control (Majumdar et al., 2015).

Carotenoids and chlorophyll assays

Carotenoids, chlorophyll *a* and *b*, and total chlorophyll contents were determined in 45 day-old plants following the Lichtenthaler and Welburn (1983) method. A sample of 0.1 g of razor blade chopped fresh leaf tissue was grinded with pestle for 5 min in a tissue grinder (Kimble Chase, 5 ml) with 5 mL of 80 % acetone. The extract was kept at – 80 °C until assays were performed. Measurements were performed using a Perkin Elmer Lambda 14 UV/Vis spectrometer at 470, 663, and 646 nm, respectively.

Catalase (CAT) analysis

Catalase (E.C. 1.11.1.6), the main H₂O₂ decomposer enzyme at peroxisome level (Scandalios, 2005), was determined in roots of 45-day old pea plants. The analysis of the catalase (CAT) activity was performed as reported by Gallego et al. (1996). For each replicate/treatment, a sample of 0.2 g of fresh tissue was finely chopped with a razor blade, grinded with mortar and pestle, and extracted with 1800 µL of phosphate buffer solution (25 mM KH₂PO₄, pH 7.4). Extracts were placed in 2 mL Eppendorf tubes and centrifuged for 10 min at -4 °C and at 9600 rpm (Eppendorf AG bench centrifuge 5417 R, Hamburg, Germany). Supernatants were transferred to clean Eppendorf tubes for the enzymatic analysis. An aliquot of 50 µL of each supernatant was transferred to a 1 mL quartz cuvette, added 950 µL of 10 mM H₂O₂, and shaken three times by hand. Absorbance was read for 3 min at 240 nm in a Perkin Elmer Lambda 14 UV/Vis Spectrometer (single-beam mode, Perkin Elmer, Uberlingen, Germany). The data was collected from four replicate/treatments.

Statistical analysis

A factorial experiment was used to perform this research. The treatments were allocated in a completely random design in the growth chamber. For all grouped comparisons against control, the data was analyzed using one-way ANOVA followed by Tukey's multiple comparisons test with a probability of $p \leq 0.05$ (Statistical Package for the Social Sciences 22, SPSS, Chicago, IL). A factorial two-way ANOVA was performed to examine the interaction between the factors: three copper compounds (three concentrations: 0, 50 and 100 mg/kg soil) and IAA (three concentrations 0, 10, and 100 μM). The Tukey HSD test was used to determine statistically significance difference between treatments means at $p \leq 0.05$.

Results and Discussions

Seed germination and plant growth

Table 1 shows the number of plants, number of leaves, root-and-stem lengths of 45-day old pea plants. Table S3 and Figure S1 show the ANOVA and bar chart for the average number of plants. As seen in Table S3, neither the Cu treatments nor the interaction $\text{Cu} \times \text{IAA}$ affected the number of plants. However, IAA showed a significant effect. The bar chart (Figure S1c) shows that IAA (average of all treatments) at 10 and 100 μM reduced the number of plants by ~23% and ~34%, respectively ($p \leq 0.05$). A previous report has shown that IAA at 100 μM combined with chromium (50-200 μM) decreased pea seed germination rate, compared with control (Gangwar and Singh, 2001).

The average number of leaf/treatments is shown in Table 1 and the statistical analysis in Table S3. As seen in Table S3 and Figure S2, there were significant effects for Cu compounds, IAA concentration, and the interaction $\text{Cu} \times \text{IAA}$. As seen in Table 1, particulate forms of Cu did not affect the number of leaves; however, ionic copper at 50 mg/kg plus IAA at 100 μM reduced the number of leaves (10 ± 0.9), compared with 100 μM IAA plus bCuO at 50 (15 ± 0.9) and 100

mg/kg (16 ± 0.9). Naeem et al. (2004) reported that in lentil (*Lens culinaris*), a close relative of green pea, IAA at 0.14 mM (140 μ M) increased the number of leaves. This suggests that an excess of exogenous IAA could counteract the possible oxidation caused by the bCuO particles (Chaoui et al. 2004). It is possible only bCuO showed significant differences due to its higher negativity (-42.7 ± 0.153 mV, pH 7), compared with nano-CuO (-34.4 ± 0.5 mV, pH 7) (Hong et al., 2015).

Table S3 shows that there were effects of Cu on stem length, while root elongation was affected by Cu and by the interaction Cu $\times$ IAA. Table 1 shows that nano-CuO at 50 mg/kg produced statistically longer stems (21.5 ± 2.0 cm) than CuCl₂ at 50 mg/kg + IAA at 100 μ M. In the case of roots, ionic Cu at 100 mg/kg + 100 μ M IAA, caused a significant reduction in root length, compared with 100 μ M IAA+ nano-CuO at both concentrations, and CuCl₂ at 50 mg/kg (Table 1). Chloride ions released by the ionization of CuCl₂ imposed significant toxicity to stems and roots of navy beans (Eaton, 1942), a member of the green pea family. Moreover, Katekar and Geissler (1982), reported that under chloride presence, pea plants form chlorinated indole acetic acid that has higher affinity than IAA for auxin receptors; thus, reducing the IAA activity in plants. In addition, Kolbert et al. (2012), reported that in *Arabidopsis thaliana*, Cu stress decreased the auxin-dependent gene expression, resulting in a reduction of root growth.

Copper concentration in green pea plant tissues

Figure 1 shows Cu concentrations in roots, stems, leaves, and pods of pea plants exposed to Cu compounds and IAA. Factorial analyses for the Cu concentrations are shown in Table S4. Although after treatment application all pots had significantly more Cu than control (Table S2), Cu accumulation in roots was significantly higher only in certain treatments. As one can see in Table S4, both the IAA and Cu compounds, as well as the interaction IAA $\times$ Cu significantly

affected the concentration of Cu in roots. Figure 1a shows that nano-CuO at 50 and 100 mg/kg, in absence of IAA, increased Cu in roots by ~136% and 130%, respectively, compared with control. Ionic Cu at 100 mg/kg plus 10 μ M IAA, also increased root Cu by 217%, compared with control. In addition nano-CuO, bCuO, and CuCl₂ at 100 mg/kg, plus 100 μ M IAA, increased root Cu by ~191%, ~197%, and ~176%, respectively, compared with control. Factor averages (IAA over Cu compounds and Cu compounds/concentrations over IAA concentrations) are shown in Figure 2a. This figure shows an increase in root Cu of 58.4% in treatments with 100 μ M IAA. Overall, Cu compounds showed statistical increases in root Cu, compared with control (Figure 2e).

Table S4 also shows that both the IAA and Cu compounds, as well as the interaction IAA $\times$ Cu significantly affected the concentration of Cu in stems. Figure 1b shows that IAA at 10 μ M, plus nano-CuO at 100 mg/kg, increased stem Cu by ~84%, while combined with bCuO at 50 mg/kg, increased stem Cu by ~78%, respectively, compared with the respective control. On the other hand, IAA at 100 μ M plus nano-CuO at 50 mg/kg increased stem Cu by ~40%, compared with control (no IAA, and 10 μ M IAA).

Factor analysis (Tables S4) shows that IAA and IAA $\times$ Cu produced significant effects on Cu accumulation in leaves. The bar chart for leaf Cu is shown in Figure 1c. As seen in this figure, only the combination of 10 μ M IAA+ nano-CuO at 100 mg/kg increased leaf Cu (57.1%), compared with the respective control. On the other hand, IAA at the two concentrations, significantly increased leaf Cu (average taken over all Cu treatments; Figure 2c). Increases were of 30% and 24% for 10 and 100 μ M, respectively, compared to controls. In the case of pods, only IAA showed significant effects on Cu accumulation (Table S3 and Figure 2d). IAA at 10 μ M increased pod Cu by ~71%, compared to controls (Figure 2d).

Copper accumulation in plants exposed to similar compounds used in this study, without the use of phytohormones, were previously reported (Wang et al., 2012; Hong et al., 2015;

Zuverza-Mena et al., 2015). Wang et al. (2012), reported 1.8 times more Cu in maize roots exposed to nano-CuO, compared with bCuO. Hong et al. (2015) exposed alfalfa and lettuce to seven Cu nanoparticles or compounds, including bCuO and nano-CuO, and reported that Cu uptake was affected by the species of plant and the Cu compound. In alfalfa, the highest Cu accumulation was found in roots and stems of plants treated with CuCl₂ at 20 mg/L, while in lettuce, the highest Cu concentration was found in roots of plants treated with CuCl₂, but in stems there was more Cu in plants treated with nano-Cu. Zuverza-Mena et al. (2015) exposed the same seven Cu nanoparticles or compounds to *Coriandrum sativum* and found that only bCuO at 20 mg/kg significantly increased shoot Cu ($p \leq 0.05$). On the other hand, the addition of IAA has shown to increase the uptake of heavy metals by plants. Lopez et al. (2005) reported that Pb uptake in alfalfa increase by 20% in plants exposed to 0.2 mM Pb/10 μ M IAA; moreover the root Pb increased to 40% when the IAA concentration increased to 100 μ M. Similar results were reported by Fassler et al. (2010) in sunflower (*Helianthus annuus*) exposed to Zn and Pb under different IAA concentrations. Pazurkiewicz- Kocot et al. (2008) mentioned that IAA activates the membrane ATPases, influencing the transport of ions resulting in influx of some elements. In addition, the Aux/IAA proteins are putative transporters (Nigam and Sawant, 2013) that could be involved in Cu accumulation and transport.

In the present study, the interaction of IAA with both bulk CuO and nano-CuO increased the translocation of Cu to stems and leaves. Moreover only IAA with nano-CuO increased leaf Cu. Although the interaction of particulate CuO with IAA is unknown, the data suggests that negatively charged CuO particles (nano and bulk) complex IAA, being subsequently transported and bound to auxin receptors in stems in leaves.

Carotenoid and chlorophyll contents

Figure 3 shows the one-way ANOVA results for carotenoids, chlorophyll *a* (chlo-a), chlorophyll *b* (chlo-b), and total chlorophyll (chlo-t), while Table S5 shows results for the factorial analysis. The factorial analysis showed that both the IAA and Cu compounds, as well as the interaction IAA $\times$ Cu compounds significantly ($p \leq 0.05$) affected carotenoid and chlorophylls. To ease the interpretation, the averages of all treatments were separated by the IAA concentration. Without IAA, CuCl₂ at 100 mg/kg decrease carotenoid in leaves by ~28%, compared to control (Figure 3a). Addition of 10 μ M IAA resulted in a reduction of ~29% and ~31% under exposure to CuCl₂ at 50 mg/kg and 100 mg/kg, respectively, compared to the respective control. Increasing the amount of IAA to 100 μ M IAA, there was less reduction (~12%), but it was still statistically significant, compared with control ($p \leq 0.05$). Since the reduction was significant without/with IAA, it can be inferred that the effects were due to the CuCl₂. Nair and Chung (2015) reported that nano-CuO significantly reduced the growth of green pea seedlings and produced lipid peroxidation. In addition, Peng et al. (2013) reported a reduction of carotenoids in *Elsholtzia splendens* (Lamiaceae) treated with 0.2 μ M CuSO₄·5H₂O.

As in the case of carotenoids, chlorophylls were significantly ($p \leq 0.05$) affected by the IAA, Cu compounds, and the interaction IAA $\times$ Cu compounds. Treatment effects on chlorophylls were somehow different, compared with carotenoids. In absence of IAA chlo-a showed reductions of ~26% and ~37% in plants exposed to bCuO 50 mg/kg and CuCl₂ 100 mg/kg, compared with control (Figure 3b) (Bernal et al., 2007). With the addition of IAA, only CuCl₂ reduced chlorophyll production. IAA at 10 μ M plus CuCl₂ at 50 and 100 mg/kg reduced chlo-a by ~37% and ~43%, respectively. Similar reductions were found when IAA was increased to 100 μ M (~39% and ~36% for 50 and 100 mg CuCl₂/kg soil, respectively) (Figure 3b). Chlo-b

showed different results compared with chl-*a*, since all treatments reduced it, compared to the absolute control (no IAA, no Cu) (Figure 3c). CuCl₂ reduced it by ~52-55%, compared with control ($p \leq 0.05$), while IAA, showed reductions of ~15% and ~29% for the 10 and 100 μ M, respectively ($p \leq 0.05$) (Figure 4c). Similar results were found for total chlorophyll (Figure 3d). Karatas et al. (2010), reported that 10 μ IAA decreased the chlorophyll and carotenoid content in *Tropaeolum majus* L. leaves. Previous studies have shown that chlorophyll is also reduced by Cu ions (Zenging, et al., 2005; Aarti et al. 2006; Perreault et al. 2010; Dimkpa et al. 2012). Kumar Shaw and Hossain (2013) informed that nano-CuO reduced carotenoids in rice (*Oryza sativa*) seedlings. Recent literature indicates that excess of Cu reduces the synthesis of protochlorophyllide reductase, which results in a reduction of chlorophyll pigments (Saglam et al., 2016). In the current study, the mixture of IAA with ionic Cu reduced chlorophyll *a* and carotenoids, but did not affect chlorophyll *b*. This could indicate that IAA protects pea plants from Cu toxicity, in terms of chlorophyll *a* production.

Enzymatic activity

The catalase (CAT) activity was recorded in roots of 45-day old plants, when the accumulation of photosynthates reaches the maximum (Figure 4). As seen in this Figure 4a, in absence of IAA, none of the Cu treatments significantly affected CAT activity. IAA alone, at 10 μ M IAA, resulted in a significant increase ($p \leq 0.05$) of CAT activity (~75%) which could be a normal response to the active cell division at root initiation induced by IAA (Figure 4b) (Buchanan et al., 2000). However, combining 10 μ M IAA with all Cu compounds/concentrations returned CAT activity to the levels of control plants (no IAA, no Cu). Similar results were found when the concentration of IAA was raised to 100 μ M. This suggests that the IAA plays a role in increasing the CAT activity, protecting plants against oxidative stress. Gangwar et al. (2012) reported that addition of

100 μ M IAA promotes the regulation of oxidative stress that metals can induce. On the other hand, the analysis of the effects of Cu shows that, except for nano-CuO at 50 mg/kg, all Cu treatments reduced CAT activity in roots (Figure 4c). Similar results were reported by Ye et al. (2014) in rice, where Cu at 50 μ M reduced CAT activity. In addition, Hong et al. (2015) reported that nano-CuO, at 20 mg/L, reduced CAT in alfalfa roots. Agami and Mohamed (2013) reported that 500 μ M IAA enhanced the antioxidant defense of wheat plants exposed to Cd stress. Dimkpa et al. (2012), reported higher CAT activity in wheat roots exposed to 500 mg/kg of nano-CuO, and Nair and Chung (2015) reported higher CAT activity in pea seedlings exposed to nano-CuO at 100 and 200 mg/kg, respectively. This suggests that, although phytohormones can mitigate the effects of metal stress, the response is linked to experimental conditions and the species of plant.

Micro- and Macro-nutrient concentrations in green pea plant tissues

Under the conditions of this study, none of the treatments affected the uptake of P and K. However, the uptake and translocation of macronutrient Ca, Mg, and S and micronutrient B, Cu, Fe, Mn, Mo, and Ni were altered in different ways (Table 2). Undisturbed plant essential elements were not included in this section. The data for Cu is shown in Figures 1 and 2 and was already discussed.

During the absorption process, only the uptake of Cu and Ca was affected by the treatments (Table 2). Copper uptake was previously discussed. The uptake of Ca significantly increased in plants exposed to ionic Cu plus IAA at 10 μ M, compared with plants exposed to 10 μ M IAA only. Previous studies have shown that IAA forms complexes with divalent metals (Ananthalakshmi et al., 2013). In addition, Zocchi and Rabotti (1993) reported increase in Ca

uptake higher than 30% in maize coleoptiles exposed o IAA at 0.1 μM . This suggests that IAA at low concentration promoted the uptake of Ca in green pea roots.

A variety of responses was observed stem samples, suggesting modifications in the translocation of essential elements. In absence of IAA, stem S and Ca increased in plants exposed to CuCl_2 at both concentrations tested. Similar results were reported by Trujillo-Reyes et al. (2014) in lettuce plants exposed to nano-Cu and nano-CuO. Shahbaz et al. (2010) reported that in Chinese cabbage exposed to 10 mM Cu^{2+} , the expression of sulfur transporter Sultr4;1 increased 4-fold S in the shoot. Trujillo-Reyes et al. (2014) reported that nano-CuO increased Ca in lettuce leaves. These authors suggested that excess Cu can damage membranes, facilitating the entrance of other elements.

With the addition of IAA at 10 μM , there was a decrease on stem Mn in plants exposed to both the nano-CuO and CuCl_2 at 100 mg/kg. Voss (1998) mention that high levels of Cu can reduce Mn, which suggests that at such Cu concentration the amount of IAA was not high enough to revert the Cu effect. When external IAA increased to 100 μM , both the stem Ca and Mg decreased in plants exposed to 100 mg/kg of either nano-CuO or bulk CuO. On the other hand, B and Mo increased. Previous studies have shown that nano-CuO at 500 mg/kg, decreased the uptake of Ca and Mn in beans (Dimkpa et al., 2015). Lopez et al. (2007b) reported that B increased in alfalfa leaves as the IAA concentration increased up to 100 μM . This could indicate a similar interaction of high IAA concentration with high concentrations of both particulate Cu compounds, perhaps due to a reaction of the negative surface charge with the IAA.

In leaves, the concentration of elements without/with IAA was well defined. Without IAA, except for B, only macroelements Ca, Mg, and S were increased, and only in plants

exposed to CuCl_2 and bCuO. With IAA, CuCl_2 reduced Fe and Ca, while both particulate compounds, bCuO and nano-CuO, increased B. Since CuCl_2 was dissolved in water previously to soil application, the response could be associated with excess of Cu or the reaction of chloride ions with soil cations. The uptake of cations by plants has been found to increase in the presence of chloride ions (Jakobsen, 1992). This suggests that in pea plants, Cl^- could have an important role in the accumulation of cations. However, reports indicates different effects of Cu in the uptake of some cations such as Mg. Panou-Filotheou et al. (2001) reported that in oregano (*Origanum vulgare* subsp. *hirtum*), Cu excess in soil resulted in an increasing of Mg in leaves. However, in hydroponically grown lettuce, nano-CuO significantly reduced Mg in leaves (Trujillo-Reyes et al. 2014). This suggests that the interaction of nano-CuO with crop plants depends on the growth media and plant species. With IAA at 100 μM , CuCl_2 at 100 mg/kg reduced Fe and Ca. Previous studies have shown that high Cu concentration reduced leaf iron by affecting ferric reductase activity (Waters and Armbrust, 2013). In addition, Cu excess has shown to reduce the accumulation of Ca in Norway spruce (Osteras and Greger, 2006). There is the possibility that the high concentration of Cu reduced the mobile phase of Fe/Ca; thus, indirectly affected their contents in plant tissues (Uchimiya et al., 2011; Tiberg et al., 2016). IAA has shown to depress nitrate reductase activity in pea roots (Sahulka, 1972) and it could influence other reductases altering the general metabolisms of pea plants. On the other hand, 100 μM IAA plus either bCuO or nano-CuO increased leaf B by more 150%, compared to IAA alone or no IAA (Table 2). Hayashi (2012) mentions that auxin-binding protein (ABP1) activates ion channels. In addition, B transport has occurs passively and trough transporters (Miwa and Fujiwara, 2010). There is the possibility that IAA activates channels for B transport or that the CuO particles activate the channels (Hughes et al., 2005).

The IAA concentrations did not affect element concentration in pods. Three elements that were increased by the Cu treatments: Ni (325%) and Fe (285%) were increased by nano-CuO at 50 mg/kg. In addition, bCuO at 100 mg/kg also increased Ni by 275.0%. Moreover, CuCl₂ increased B by ~47, compared to control. Compartmentation of elements to pods depends on the phloem loading, which, in some cases, like in the case of Fe, depends on specific chelators (Grusak et al. 1999). So far, there is no data to explain why particulate CuO promoted the increased concentration of Fe, Ni, and B in pea pods. More research is needed to explain these results.

Dry biomass production

The root biomass was not calculated because in some treatments, even flooded for about 15 min, it was not possible to recover small roots from some soil fragments. The dry biomass of stems, leaves, and pods is shown in Table 3. As seen in this table, there were no differences in leaves biomass, but pods and stems showed significant differences, compared with controls. The IAA *per se* (Table 3, treatment ID # 1, 8, and 15), did not significantly affect the biomass production in leaves and pods. However, combined with some Cu compounds altered the biomass production. In pods, for instance, IAA at 10 μ M combined with nano-CuO at 50 mg/kg and CuCl₂ at 100 mg/kg reduced the biomass by about 50%. Since the reduction was similar for both the nanoparticulate and ionic Cu, it is possible that the effect was due to the IAA, not to the interaction of IAA with the Cu compounds; however, more investigation is needed in order to clarify this assumption. In stems, none of the treatments that included IAA at 10 μ M altered the biomass; however, 100 μ M IAA combined with nano-CuO at 50 mg/kg significantly reduced the biomass, while combined with bCuO at 100 mg/kg increase it by more than 60%, compared with controls. There is no information concerning the interaction of

auxins with NPs and controversies in the response of plants to nano-CuO. Dimkpa et al. (2012) reported an increase in wheat biomass under exposure to nano-CuO at 500 mg/kg for 14 days, which was not observed under bCuO exposure. Dimkpa et al. (2012) also reported that both the nano-CuO and bCuO released similar amount of Cu ions (about 3 mg/L); suggesting that the effects were not associated with the ionic Cu but with the particle size. Conversely, Nair et al. (2015) reported that nano-CuO in the range of 100-500 mg/L, reduced green pea plant growth, while Gangwar et al. (2011), reported that 100 μ M IAA decrease pea seedlings growth. However, the lack of studies involving both exogenous IAA and nano-CuO or bCuO does not allow comparisons of the results with previous reports.

Conclusions

Literature on effects of the interaction among NPs and phytohormones in plants is scarce. In this study, green pea plants were cultivated in natural soil amended with either nano-CuO, bulk-CuO or CuCl₂ plus IAA at 10 and 100 μ M. The factorial analysis show that IAA reduced the number of plants. In addition, IAA combined with ionic Cu reduced the number of leaves, stem, and root lengths. The interaction of IAA with Cu compounds also reduced carotenoids and chlorophyll production. Moreover, IAA at 10 μ M plus nano-CuO at 50 mg/kg reduced stem and pod biomass. All the treatments affected the concentration of micro- and macroelements in tissues. In general the highest Cu treatment concentration (100 mg/kg) increased Cu in roots. The concentration of essential elements, in response to IAA treatments varied with the tissues. The auxin alone or combined with Cu NPs/compounds did not affect pod element concentration. However, they increased Ca in roots, B in stem and leaf but reduced Mg, Ca, and Mn in stem and Fe and Ca in leaves. On the other hand, without IAA CuCl₂ increased B, Ca, Mg, and S in stems and Ca and B in leaves. Finally, nano-CuO at 50 mg/kg increased pod Fe and Ni; bCuO at 100 mg/kg also

increased pod Ni, while CuCl₂ at both concentrations increased pod B. This suggests that the Cu NPs or Cu compounds, alone or combined with IAA alter plant ionome. However, results indicate that IAA plus CuCl₂ imposed higher toxicity. Studies with different concentrations of phytohormones, Cu NPs, or Cu compounds, and in soils with different properties are needed in order to better understand the effects of these amendments in green pea plants.

Acknowledgements

This material is based upon work supported by the National Science Foundation and the Environmental Protection Agency under Cooperative Agreement Number DBI-1266377. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation or the Environmental Protection Agency. This work has not been subjected to EPA review and no official endorsement should be inferred. The authors also acknowledge the USDA grant 2016-67021-24985 and the NSF Grants EEC-1449500, CHE-0840525 and DBI-1429708. Partial funding was provided by the NSF ERC on Nanotechnology-Enabled Water Treatment (EEC-1449500). This work was also supported by Grant 2G12MD007592 from the National Institutes on Minority Health and Health Disparities (NIMHD), a component of the National Institutes of Health (NIH). J. L. Gardea-Torresdey acknowledges the Dudley family for the Endowed Research Professorship and the Academy of Applied Science/US Army Research Office, Research and Engineering Apprenticeship program (REAP) at UTEP, grant #W11NF-10-2-0076, sub-grant 13-7, and the LERR and STARs programs of the University of Texas System.

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Figure 1. Copper concentration in (a) roots, (b) stems, (c) leaves of green pea plants grown to 45 days (beginning of blossom), and (d) pods of plants grown for 90 days (full maturity) in soil amended with 50 and 100 mg/kg of Cu from nano-CuO, bCuO, and CuCl₂ and IAA at 0, 10, and 100 μ M. Data are means $\pm$ SE of four replicates. Bars with different letters are statistically different at $p \leq 0.05$. * Indicate statistical differences compared to their respective control.

Figure 2. Average Cu concentration in roots, stems, leaves, and pods as affected by IAA concentrations (a-d) and copper compounds/concentrations (e-h). Plants were cultivated in soil amended with 50 and 100 mg/kg of Cu from nano-CuO, bCuO, and CuCl₂ and IAA at 0, 10, and 100 μ M. Data are means $\pm$ SE of four replicates. Bars with different letters are statistically different at $p \leq 0.05$.

Figure 3. (a) Carotenoids, (b) chlorophyll *a*, (c) chlorophyll *b* and (d) total chlorophyll in leaves of 45 day-old plants grown in soil amended with 50 and 100 mg/kg of Cu from nano-CuO, bCuO, and CuCl₂ and IAA at 0, 10, and 100 μ M. Data are means $\pm$ SE of four replicates. Bars with different letters are statistically different at $p \leq 0.05$. * Indicate statistical differences compare to their respective control.

Figure 4. (a) CAT enzymatic activity in green pea roots from plants grown for 45 days in soil amended with 50 and 100 mg/kg of Cu from nano-CuO, bCuO, and CuCl₂ and IAA at 0, 10, and 100 μ M. (b) Effects of IAA concentrations and (c) effects of Cu compounds/concentrations. Data are means $\pm$ SE of four replicates. Bars with different letters are statistically different at $p \leq 0.05$.

Table 1. Number of plants, root and stem elongation, and number of leaves/plant of green pea cultivated in soil amended with 50 and 100 mg/kg Cu from nano-CuO, bulk-CuO, and CuCl₂ and IAA at 10 and 100 μ M. Results are means $\pm$ SE of four replicates. Letters indicate statistical differences ($p \leq 0.05$).

Treatments			Growth Response			
ID	IAA (μ M)	Cu Compound (mg/kg of soil)	Stem length (cm)	Root length (cm)	Number of leaves	Number of plants
1	0	0	17.6 $\pm$ 1.6 ab	23.0 $\pm$ 2.0 abc	13 $\pm$ 0.6 abcd	4 $\pm$ 0.4 ab
2		n50	21.5 $\pm$ 2.1 a	26.1 $\pm$ 2.8 ab	14 $\pm$ 0.1 abcd	3.5 $\pm$ 0.3 ab
3		n100	16.1 $\pm$ 0.8 ab	27.8 $\pm$ 1.0 ab	12 $\pm$ 0.3 abcd	4.5 $\pm$ 0.5 a
4		b50	16.2 $\pm$ 2.3 ab	27.7 $\pm$ 3.3 ab	12 $\pm$ 1.2 abcd	4.25 $\pm$ 0.8 ab
5		b100	16.5 $\pm$ 1.0 ab	24.3 $\pm$ 4.4 abc	12 $\pm$ 0.6 abcd	4.25 $\pm$ 0.3 ab
6		CuCl ₂ 50	16.1 $\pm$ 3.0 ab	19.1 $\pm$ 1.3 bc	11 $\pm$ 1.1 abcd	4.25 $\pm$ 0.3 ab
7		CuCl ₂ 100	14.1 $\pm$ 1.0 ab	19.4 $\pm$ 3.2 bc	10 $\pm$ 0.5 bcd	4.25 $\pm$ 0.5 ab
8	10	0	17.0 $\pm$ 4.0 ab	19.5 $\pm$ 2.4 bc	11 $\pm$ 1.0 abcd	3 $\pm$ 0.4 ab
9		n50	19.5 $\pm$ 1.8 ab	23.0 $\pm$ 2.0 abc	12 $\pm$ 0.7 abcd	3 $\pm$ 0.0 ab
10		n100	13.7 $\pm$ 1.9 ab	24.3 $\pm$ 2.3 abc	10 $\pm$ 0.7 cd	3.25 $\pm$ 0.6 ab
11		b50	15.4 $\pm$ 2.1 ab	22.1 $\pm$ 1.5 bc	12 $\pm$ 0.6 abcd	2.5 $\pm$ 0.3 ab
12		b100	17.1 $\pm$ 1.3 ab	18.6 $\pm$ 1.1 bc	14 $\pm$ 1.0 abcd	4.25 $\pm$ 0.3 ab
13		CuCl ₂ 50	17.4 $\pm$ 2.6 ab	23.8 $\pm$ 2.4 abc	13 $\pm$ 1.4 abcd	2.75 $\pm$ 0.9 ab
14		CuCl ₂ 100	13.8 $\pm$ 1.3 ab	23.9 $\pm$ 0.7 abc	11 $\pm$ 0.6 abcd	3.5 $\pm$ 0.3 ab
15	100	0	16.9 $\pm$ 0.8 ab	23.4 $\pm$ 1.1 abc	13 $\pm$ 0.5 abcd	3.25 $\pm$ 0.9 ab
16		n50	16.6 $\pm$ 3.4 ab	30.5 $\pm$ 2.1 ab	13 $\pm$ 1.2 abcd	1.75 $\pm$ 0.3 b
17		n100	17.6 $\pm$ 1.1 ab	35.1 $\pm$ 4.2 a	15 $\pm$ 1.2 abc	2.5 $\pm$ 0.6 ab
18		b50	16.4 $\pm$ 1.6 ab	23.2 $\pm$ 3.7 abc	15 $\pm$ 1.3 ab	3.75 $\pm$ 0.3 ab
19		b100	16.5 $\pm$ 0.6 ab	24.9 $\pm$ 0.8 abc	16 $\pm$ 1.7 a	2.5 $\pm$ 0.3 ab
20		CuCl ₂ 50	10.7 $\pm$ 1.1 b	26.1 $\pm$ 1.9 ab	10 $\pm$ 0.9 d	2.25 $\pm$ 0.6 ab
21		CuCl ₂ 100	12.9 $\pm$ 1.4 ab	12.9 $\pm$ 1.4 c	11 $\pm$ 0.7 abcd	3.25 $\pm$ 0.5 ab

Table 2. Micro- and Macro- nutrients affected in organs of green pea plants grown for 45 days in natural soil amended with 50 and 100 mg/kg of Cu from nano-CuO, bulk CuO, and CuCl₂ plus indole-3-acetic acid (IAA) at 10 and 100 µM. The data from pods was obtained from 90-day old plants. Data are averages ± SE of four replicates. Comparisons were made with the respective controls ($p \leq 0.05$). Arrows indicate increase or decrease.

Plant Part	Element Affected	Treatment	Concentration (mg/kg d wt tissue)	%	
Pods	Fe	Control (0 µM IAA)	11.9 ± 2.9	100.00%	-
		nCuO 50 mg/kg	42.6 ± 9.7	258.00%	↑
	B	Control (0 µM IAA)	25.9 ± 3.1	100.00%	-
		CuCl ₂ 50 mg/kg	38.2 ± 1.6	47.50%	↑
		CuCl ₂ 100 mg/kg	38 ± 3.5	46.70%	↑
	Ni	Control (0 µM IAA)	0.4 ± 0.1	100.00%	-
		nCuO 50 mg/kg	1.7 ± 0.4	325.00%	↑
		bCuO 50 mg/kg	1.5 ± 0.3	275.00%	↑
Leaves	Fe	Control (100 µM IAA)	117.5 ± 13.9	100.00%	-
		100 µM IAA + CuCl ₂ 50 mg/kg	69.6 ± 3.1	40.80%	↓
		100 µM IAA + CuCl ₂ 100 mg/kg	58.7 ± 2.7	50.00%	↓
	B	Control (0 µM IAA)	2.0 ± 0.9	100.00%	-
		CuCl ₂ 100 mg/kg	19.7 ± 4.9	885.00%	↑
		Control (100 µM IAA)	28.8 ± 6.5	100.00%	-
		100 µM IAA + nCuO 50 mg/kg	78.8 ± 15.9	173.60%	↑
		100 µM IAA + nCuO 100 mg/kg	92.2 ± 1.9	220.10%	↑
		100 µM IAA + bCuO 50 mg/kg	96.2 ± 6.0	234.00%	↑
		100 µM IAA + bCuO 100 mg/kg	111.7 ± 8.0	287.80%	↑
	Ca	Control (0 µM IAA)	35226 ± 1282	100.00%	-
		bCuO 50 mg/kg	50860 ± 4071	44.40%	↑
		bCuO 100 mg/kg	51215 ± 1497	45.40%	↑
		CuCl ₂ 50 mg/kg	52008 ± 4279	47.60%	↑
		CuCl ₂ 100 mg/kg	60660 ± 2787	72.20%	↑
		Control (100 µM IAA)	51861 ± 2821	100.00%	-
	Mg	100 µM IAA + CuCl ₂ 100 mg/kg	33013 ± 1891	36.30%	↓
		Control (0 µM IAA)	4506 ± 10.1	100.00%	-
		bCuO 50 mg/kg	6041 ± 335.4	34.10%	↑
		bCuO 100 mg/kg	6125 ± 264.7	35.90%	↑
		CuCl ₂ 50 mg/kg	6336 ± 474.4	40.60%	↑
		CuCl ₂ 100 mg/kg	6830 ± 252.0	51.60%	↑

Stems		Control (100 μ M IAA)	5684 ± 361.2	100.00%	-
		100 μ M IAA + nCuO 50 mg/kg	7095 ± 312.4	24.80%	↑
	S	Control (0 μ M IAA)	2623 ± 200.7	100.00%	-
		CuCl ₂ 50 mg/kg	8328 ± 1060.6	217.50%	↑
		CuCl ₂ 100 mg/kg	9554 ± 1818.4	264.20%	↑
	Mn	Control (10 μ M IAA)	16.1 ± 0.5	100.00%	-
		10 μ M IAA + nCuO 100 mg/kg	13.1 ± 0.4	18.60%	↓
		10 μ M IAA + CuCl ₂ 100 mg/kg	12.6 ± 0.9	21.70%	↓
	B	Control (100 μ M IAA)	3.2 ± 1.1	100.00%	-
		100 μ M IAA + bCuO 50 mg/kg	11.7 ± 0.8	265.60%	↑
		100 μ M IAA + bCuO 100 mg/kg	10.3 ± 1.5	221.90%	↑
		100 μ M IAA + CuCl ₂ 100 mg/kg	10.8 ± 0.4	237.50%	↑
	Mo	Control (100 μ M IAA)	44.6 ± 8.6	100.00%	-
		100 μ M IAA + bCuO 100 mg/kg	82.5 ± 8.6	85.00%	↑
	Ca	Control (0 μ M IAA)	14671 ± 1540	100.00%	-
		CuCl ₂ 50 mg/kg	19478 ± 88	32.80%	↑
		CuCl ₂ 100 mg/kg	23920 ± 1372	63.00%	↑
		Control (100 μ M IAA)	18121 ± 605	100.00%	-
		100 μ M IAA + nCuO 100 mg/kg	12328 ± 937	32.00%	↓
		100 μ M IAA + bCuO 100 mg/kg	11389 ± 568	37.20%	↓
	Mg	Control (100 μ M IAA)	5823 ± 361	100.00%	-
		100 μ M IAA + nCuO 100 mg/kg	3757 ± 254	35.50%	↓
		100 μ M IAA + bCuO 100 mg/kg	3767 ± 261	35.30%	↓
	S	Control (0 μ M IAA)	3676 ± 129	100.00%	-
		CuCl ₂ 50 mg/kg	7059 ± 593	92.00%	↑
		CuCl ₂ 100 mg/kg	7998 ± 1212	117.60%	↑
Roots	Ca	Control (10 μ M IAA)	12064 ± 749	100.00%	-
		10 μ M IAA + CuCl ₂ 50 mg/kg	17823 ± 738	47.70%	↑
		10 μ M IAA + CuCl ₂ 100 mg/kg	19559 ± 1568	62.10%	↑

Table 3. Dry biomass (g) of leaves, and stems of green pea plants grown for 45 days in soil amended with nano-CuO, bulk CuO and CuCl₂ at 0-100 mg/kg and IAA at 0-100 μ M. The biomass of pods was recorded at 90 days. Results are means $\pm$ SE of four replicates. Letters indicate statistical differences between treatments and * indicates statistical differences compared to the respective control ($p \leq 0.05$).

ID	IAA (μ M)	Cu Compound (mg/kg)	Dry biomass tissue (g)		
			Pods	Leaves	Stems
1	0	0	0.72 $\pm$ 0.07 abc	1.01 $\pm$ 0.08 a	0.25 $\pm$ 0.03 abc
2		n50	0.52 $\pm$ 0.10 abcde	0.94 $\pm$ 0.06 a	0.29 $\pm$ 0.04 ab
3		n100	0.60 $\pm$ 0.05 abcde	0.93 $\pm$ 0.11 a	0.27 $\pm$ 0.02 ab
4		b50	0.52 $\pm$ 0.06 abcde	0.71 $\pm$ 0.05 a	0.22 $\pm$ 0.01 bc
5		b100	0.62 $\pm$ 0.05 abcde	0.94 $\pm$ 0.06 a	0.27 $\pm$ 0.01 ab
6		CuCl ₂ 50	0.64 $\pm$ 0.04 abcde	0.86 $\pm$ 0.08 a	0.24 $\pm$ 0.05 abc
7		CuCl ₂ 100	0.74 $\pm$ 0.04 ab	0.72 $\pm$ 0.09 a	0.21 $\pm$ 0.02 bc
8	10	0	0.37 $\pm$ 0.10 cde	0.58 $\pm$ 0.14 a	0.15 $\pm$ 0.02 bc
9		n50	0.35 $\pm$ 0.07 de	0.67 $\pm$ 0.08 a	0.17 $\pm$ 0.04 bc
10		n100	0.56 $\pm$ 0.06 abcde	0.56 $\pm$ 0.17 a	0.18 $\pm$ 0.05 bc
11		b50	0.38 $\pm$ 0.04 cde	0.52 $\pm$ 0.07 a	0.15 $\pm$ 0.02 bc
12		b100	0.44 $\pm$ 0.05 bcde	0.81 $\pm$ 0.08 a	0.23 $\pm$ 0.03 bc
13		CuCl ₂ 50	0.44 $\pm$ 0.09 abcde	0.93 $\pm$ 0.17 a	0.29 $\pm$ 0.04 ab
14		CuCl ₂ 100	0.33 $\pm$ 0.02 e	0.96 $\pm$ 0.10 a	0.30 $\pm$ 0.04 ab
15	100	0	0.59 $\pm$ 0.06 abcde	0.56 $\pm$ 0.12 a	0.21 $\pm$ 0.02 bc
16		n50	0.60 $\pm$ 0.06 abcde	0.48 $\pm$ 0.06 a	0.09 $\pm$ 0.02 c
17		n100	0.66 $\pm$ 0.03 abcde	0.85 $\pm$ 0.11 a	0.27 $\pm$ 0.03 ab
18		b50	0.80 $\pm$ 0.06 a	0.90 $\pm$ 0.04 a	0.30 $\pm$ 0.01 ab
19		b100	0.73 $\pm$ 0.01 abc	1.02 $\pm$ 0.09 a	0.41 $\pm$ 0.02 a*
20		CuCl ₂ 50	0.73 $\pm$ 0.11 abc	0.50 $\pm$ 0.21 a	0.18 $\pm$ 0.07 bc
21		CuCl ₂ 100	0.70 $\pm$ 0.12 abcd	0.92 $\pm$ 0.05 a	0.25 $\pm$ 0.02 abc

Figure 1

Figure 2

Figure 3

Figure 4