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Improvement of nutrient elements and allicin content in green onion (*Allium fistulosum*) plants exposed to CuO nanoparticles

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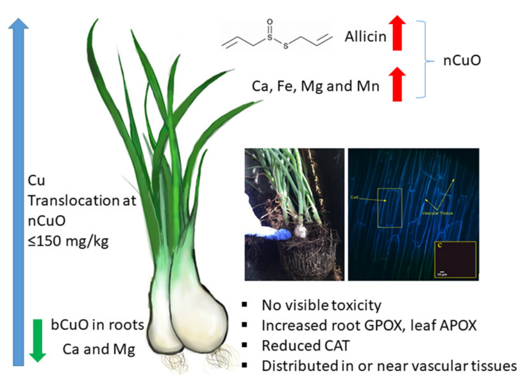
HIGHLIGHTS

- None of the Cu-based compounds produced visible signs of toxicity.
- Allicin, Ca, Fe, Mg, and Mn contents were improved by nCuO, but not by bCuO.
- Nano CuO increased root GPOX and leaf APOX, but reduced CAT in roots and leaves.
- Cu was translocated to leaves dependent on the Cu compound exposure manner.
- Cu uptake distribution from bCuO and nCuO treatments indicated an apoplastic pathway.

GRAPHICAL ABSTRACT



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ABSTRACT

With the exponential growth of nanomaterial production in the last years, nano copper (Cu)-based compounds are gaining more consideration in agriculture since they can work as pesticides or fertilizers. Chinese scallions (*Allium fistulosum*), which are characterized by their high content of the antioxidant allicin, were the chosen plants for this study. Spectroscopic and microscopic techniques were used to evaluate the nutrient element, allicin content, and enzyme antioxidant properties of scallion plants. Plants were harvested after growing for 80 days at greenhouse conditions in soil amended with CuO particles [nano (nCuO) and bulk (bCuO)] and $CuSO_4$ at 75–600 mg/kg]. Two-photon microscopy images demonstrated the particulate Cu uptake in nCuO and bCuO treated roots. In plants exposed to 150 mg/kg of the Cu-based compounds, root Cu content was higher in plants treated with nCuO compared with bCuO, $CuSO_4$, and control ($p \leq 0.05$). At 150 mg/kg, nCuO increased root Ca (86%), root Fe (71%), bulb Ca (74%), and bulb Mg (108%) content, compared with control ($p \leq 0.05$). At the same concentration, bCuO reduced root Ca (67%) and root Mg (33%), compared with control ($p \leq 0.05$). At all concentrations, nCuO and $CuSO_4$ increased leaf allicin (56–187% and 42–90%, respectively), compared with control

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Stress enzymes
Allicin

($p \leq 0.05$). The antioxidant enzymes were differentially affected by the Cu-based treatments. Overall, the data showed that nCuO enhances nutrient and allicin contents in scallion, which suggests they might be used as a nanofertilizer for onion production.

1. Introduction

Metallic engineered nanoparticles (ENPs), such as nano silver (nAg), nano copper (Cu) oxide (nCuO), nano copper hydroxide ($nCu(OH)_2$), and nano cerium oxide ($nCeO_2$), have promising use in the agriculture (Iavicoli et al., 2017; Prasad et al., 2017; Servin and White, 2016; White and Gardea-Torresdey, 2018). They can benefit agricultural productivity through controlling pests, supplying nutrients, and suppressing plant diseases (White and Gardea-Torresdey, 2018). It is estimated that from total nanoproducts production, nearly 9% may be used in agriculture in the form of nanopesticides, nanoherbicides, and nanofertilizers, among others (Iavicoli et al., 2017; Rai and Ingle, 2012; White and Gardea-Torresdey, 2018).

Nanoscale Cu materials reached a total global production of 200 tons in 2010 (Keller et al., 2013). The global market of nCuO was $24.66 million in 2015, and expected to reach $120.67 million by 2022 ("Nano Copper Oxide Market Size and Industry Forecast - 2022", 2016). Recently, nCuO was reported to be a potential pesticide to deal with fungi and bacteria (Adisa et al., 2019; Huang et al., 2019; Zhang et al., 2018). As a result, the amount of nCuO in the environment may increase. Previous studies on the interaction of nCuO with plants showed species-dependent results (Du et al., 2019; Fatima et al., 2020; Shang et al., 2019). For instance, the growth of duckweed (*Lemna minor*) was restrained after seven days of hydroponic exposure to nCuO at 150 μg/L (Yue et al., 2018). Chloroplast and starch grain shrinkage were also found. However, a full life cycle study on soil-grown bell pepper (*Capsicum annuum* L.) revealed that nCuO and bulk CuO particles (bCuO), at 125 to 500 mg/kg, did not change dry plant biomass, foliar area, leaf pigments, and fruit yield (Rawat et al., 2018).

Two-photon microscopy has shown many advantages in detecting ENPs in biological samples (Bonilla-Bird et al., 2018). Compared with the scanning electron microscope or confocal microscopy, it is capable of penetrating the sample and obtaining images deep from the surface up to 1 mm. As previously shown, nCuO exhibited autofluorescence by two-photon laser excitation (Bonilla-Bird et al., 2018), thus, these nanoparticles (NPs) are good candidates to explore their uptake mechanism by plant cells.

Scallion or green onion (*Allium fistulosum*), is used as an important seasoning worldwide (Tabassum et al., 2016). They have a high concentration of allicin, flavonoids, vitamins, as well as other organosulfur compounds (Yin et al., 2003). Allicin, one of the most distinctive organosulfur compounds, is responsible for the pungency and flavor in *Allium* monocotyledonous flowering plants (Block et al., 1992). Additionally, scallions pose antioxidant and antibacterial properties and have shown potential to benefit the cardiovascular system (Chan et al., 2013). In 2007, the top five producers countries (Japan, China, Republic of Korea, Nigeria, and New Zealand) registered more than three million metric tons of scallions (The Top 5 Green Onion Producing Countries, 2007).

However, the scallion is not a very well-studied species in the *Allium* family. To the authors' best knowledge, only one study has been focused to evaluate the effects of ENPs on scallion plants. This mentioned study reported an antimycotic effect of nAg against the fungus *Sclerotium cepivorum* that impairs green onion plants (Jung et al., 2010). However, the uptake of ENPs by scallion plants, the effects of ENPs on the nutrient elements, allicin, and antioxidant species are still unknown. Thus, the aim of this study was to perform a full-lifecycle study to examine the effect of Cu-based compounds on the nutrient element behavior, allicin content, and enzyme antioxidant responses in scallion plants. The Cu uptake and nutrient element accumulation on different parts of scallion were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES). The root samples were screened by two-photon microscopy to observe the penetration and retention of nCuO and bCuO. To the authors' knowledge, this is the first report that describes the effects of ENPs on allicin accumulation in plants.

2. Materials and methods

2.1. Preparation of $nCuO_2$ amended soil

The nCuO were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC-CEIN). Copper sulfate ($CuSO_4$) and bCuO were purchased from Sigma Aldrich (St. Louis, MO). Hong et al. previously reported the characterization data of nCuO and bCuO, which can be found in Table S1 (Hong et al., 2015).

A mixing ratio of 2:1 by weight between nature soil and potting soil was used to ensure optimum plant growth. Commercial Miracle-Gro® organic potting soil was purchased from a local supermarket. Natural soil was collected at Socorro TX, USA, an agricultural land at latitude: 31°67′ N, longitude: 106°28′ W, and elevation: 1115 *m* asl. The soil was characterized previously as medium loam (19% clay, 44% silt, and 36% sand; 2.8% organic matter, pH $= 7.8 \pm 0.02$, EC $= 1705 \pm 47.6$ μS cm^{-1}, and TDS $= 847.5 \pm 23.8$ mg/L) (Majumdar et al., 2015). To remove plant-based residues and gravel, the soil collected was sieved through an 8 mm sieve, and air-dried for two days.

Before the soil amendment, suspensions/solutions of each of the three Cu-based compounds (nCuO, bCuO, and $CuSO_4 5H_2O$) were made to obtain 75, 150, 300, and 600 mg Cu content/kg soil. Environmentally relevant concentrations of Cu compounds were selected for the current study. For example, the lowest concentration was selected according to the highest Cu content reported in agricultural soil amended with sewage sludge (75 mg/kg) (McGrath et al., 1994). Briefly, nCuO, bCuO, and $CuSO_4$ were weighted, suspended in 50 mL Milli-Q 18 MΩ deionized water (DI), and sonicated in a water bath at 25 °C for 30 min and 180 watts. The 1.5 kg soil was then amended with suspensions and mixed manually for 20 min until homogenous. The control soil was prepared with 50 mL of DI. The treatments were then filled with DI to 60% of the maximal field capacity and prepared in triplicate. All the pots were allowed to equilibrate for three days before use.

2.2. Seed germination and plant growth

Scallion seeds (evergreen bunching) were purchased from the Ferry-Morse Seeds Company and stored at 4 °C. Seeds were rinsed with 2% NaClO for 15 min and washed three times with DI. For hydration, seeds were soaked with DI for 12 h and sowed in germination soil. Two weeks after sowing, seedlings were transplanted into pots containing the Cu-treatments or control and placed in the greenhouse with temperature setpoint at 30/20 °C day/night temperature 50–75% relative humidity, and daily light integral of 10 mol m^{-2} d^{-1} on average. All pots were irrigated and fertilized with 100 mL per day of water-soluble fertilizer 15 N–2.2P–12.5 K (Peters 15–5-15 Cal-Mag Special; Scotts, Marysville, OH) at 1.0 $g \cdot L^{-1}$. Plants were harvested 80 days after transplanting, washed with 0.01% HNO_3 and DI for three times and prepared for different analyses.

2.3. Chlorophyll measurement

Fresh leaf samples (~0.5 g) were ground and the chlorophyll was extracted with 80% acetone. A UV–Vis Spectrophotometer (Perkin Elmer Lamda, Uberlinger, Germany) was used to measure the absorbance as described by Porra (Porra, 2002).

2.4. Quantification of allicin

Allicin content was determined using a UV–Vis Spectrophotometer (Perkin Elmer Lamda) according to Han et al. (Han et al., 1995). Briefly, ~0.3 g of dry green onion leaf tissues were ground and dissolved in 150 mL of DI. After centrifugation, only the supernatants remained. A mixture of 0.1 mL of the extract and 0.24 mL of 2 mM L-cysteine was kept at room temperature for 10 min. After adding 0.6 mL of 50 mM HEPES buffer (pH 7.6) and 0.2 mL DTNB, the mixture was shaken for 2 min and read at 412 nm using UV–Vis Spectrophotometer.

2.5. Antioxidant enzymatic activities

A UV–Vis Spectrophotometer (Perkin Elmer Lamda) was used to determine the enzyme activities. Antioxidant enzymatic activities of catalase (CAT), ascorbate peroxidase (APOX), guaiacol peroxidase (GPOX), and superoxide dismutase (SOD) were analyzed to understand scallion antioxidant defense system under the interaction of Cu based compounds. All the analyses were done at 25 °C. The crude extraction for CAT and SOD was prepared following Lee et al. (Lee and Lee, 2000). The CAT and SOD activity was determined according to Aebi (Aebi, 1974), and Beyer and Fridovich (Beyer and Fridovich, 1987), respectively. APOX and GPOX extractions were prepared according to Xu et al. (Xu and Chen, 2011). The APOX and GPOX activity was determined following Nakano and Asada (Nakano and Asada, 1981), and Egley (Egley et al., 1983), respectively.

2.6. Essential element quantification by ICP-OES and two-photon microscopy sample preparation

Macronutrients (Ca, Mg, P, S, and K) and micronutrients (Cl, Zn, Fe, B, Mn, Cu, Mo, and Ni) were measured by ICP-OES (Perkin-Elmer Optima 4300 DV; Shelton, CT). Plants were severed into roots, bulbs, and leaves. Each sample was oven-dried at 70 °C for 72 h. A coffee grinder (Hamilton Beach) was used to homogenize dry samples. Around 0.2 g of dry tissues were acid-digested after adding 2 mL of pure trace HNO_3 (SPC Science, Champlain, NY), at 115 °C for 45 min in a Digiprep hot block (SCP Science). To digest completely, another 20 min incubation was applied with an additional 1 mL of 30% hydrogen peroxide. The digested solutions were adjusted to 45 mL with DI. To validate the measurements, blanks (no plant tissues), standard reference material (peach leaf, 1547, National Institute of Standards and Technology, Gaithersburg, MD) and spiked samples of 10 and 50 mg/kg were also treated by the same procedure. For QC, the multi-elemental standard solution was evaluated every 20 samples. The recovery rate was 99%.

For two-photon microscope imaging, fresh root samples of 80-day-old scallion control and treatment exposed to nCuO at 600 mg/kg soil was collected and cut to thin slices using Microtome. Then, the samples were analyzed by a mode-locked Cu: Sapphire laser (Spectra-Physics, Mai-Tai HP) at 710 nm light and 200 W. A 665 nm long-pass dichroic mirror was used to deflect the fluorescence signal from the sample. In the end, two-dimensional images were acquired using a custom software program (Acosta et al., 2014).

2.7. Statistical analysis of the data

All the data were analyzed by the Statistical Package for the Social Sciences 25 (SPSS, Chicago, IL, USA). Statistical significance was accepted at a p-value of 0.05. Data are mean ± standard error (SE) of three replicates as stated in tables/figures. One-way ANOVA followed by the Tukey–Kramer multiple comparison tests were used to determine the differences among treatment means.

3. Result and discussion

3.1. Uptake of nCuO and bCuO particles

The Cu uptake in the roots of plants treated with nCuO and bCuO was detected using two-photon microscopy (Fig. 1). This was also quantitatively corroborated by the ICP-OES. Fig. 1d and f show fluorescent points from root tissue exposed to bCuO and nCuO, respectively, at 600 mg/kg. Representative spots are pointed out by yellow arrows and squares as examples. These glittering points are similar to those of the pure compounds suspended in DI (Fig. 1e and g); thus, they are suggested to belong to bCuO (Fig. 1d) and nCuO (Fig. 1f). Both bCuO and nCuO were mainly distributed in or near the vascular tissues and in the intercellular spaces of roots, indicating an apoplastic uptake. Accordingly, Bonilla et al. previously showed that bCuO and nCuO compounds were accumulated in intercellular spaces of sweet potato roots (Bonilla-Bird et al., 2018).

Conversely, Fig. 1a and b are representative images of root samples of plants exposed to H_2O (control) and $CuSO_4$ at 600 mg/kg, respectively. In these micrographs, parameters were set to detect fluorescence from nCuO/bCuO, but no flashing or bright signal was found. Barely, structures of root cells and vascular tissue with typical autofluorescence were only encountered. Besides, the image of $CuSO_4$ dissolved in DI water at 600 mg Cu/L is shown in Fig. 1c. No visible fluorescence of ion Cu^{2+} is seen due to the dissolution.

3.2. Copper content in roots, bulbs, and leaves

In roots, all Cu-based treatments at ≥300 mg/kg significantly increased Cu compared with control (Fig. 2a) ($p \le 0.05$). Both bCuO and $CuSO_4$ at 75 and 150 mg/kg, did not affect root Cu accumulation, compared with control. Meanwhile, nCuO at 150 mg/kg increased root Cu by 9.5-fold compared with control ($p \le 0.05$). It is worth noting that at 150 mg/kg, nCuO treatment significantly increased root Cu concentration compared with bCuO and $CuSO_4$ (90.0 vs. 33.8 and 43.7 mg/kg dry weight (DW), respectively) ($p \le 0.05$).

Fig. 2b shows that at ≥300 mg/kg, all Cu-based compounds significantly increased bulb Cu (11–17 mg/kg DW), compared with control (4.5 mg/kg DW) ($p \le 0.05$). Moreover, when plants were treated with ≤150 mg/kg Cu compounds, the nCuO treatment did not significantly increase Cu content in the bulb compared with control, as was predominantly exhibited by bCuO and $CuSO_4$ (Fig. 2b).

Fig. 2c shows that Cu accumulation in leaves was significantly increased in plants treated with nCuO at ≤150 mg/kg and with $CuSO_4$ at ≥150 mg/kg. When comparing the effect of different compounds at 75 mg/kg, the results showed that nCuO induced a greater Cu accumulation in leaves than bCuO and $CuSO_4$ (31 vs. 15 and 12 mg/kg DW, respectively) ($p \le 0.05$). In contrast to $CuSO_4$, which dissolves easily, and its ions can actively move through ion transporters, nCuO and bCuO exhibit slow Cu release. However, nCuO and bCuO may be taken by roots in particulate forms, as confirmed by the two-photon images (Fig. 1d and f). Interestingly, at 75 mg/kg, a possible combination of less aggregation, smaller particle size, and a higher dissolution rate of the nCuO treatment enabled the higher accumulation of Cu in leaves, compared with bCuO and $CuSO_4$ (Fig. 2c). With the increasing concentration of nCuO, the characteristics mentioned above were no longer obvious due to the apparent aggregation of nCuO. Similar results were reported before in nCuO or bCuO treated bell pepper (*Capsicum annum* L.) and sugarcane (*Saccharum officinarum*) plants (Rawat et al., 2018; Tamez et al., 2019). Additionally, released ions from particulate compounds might be involved in the Cu uptake.

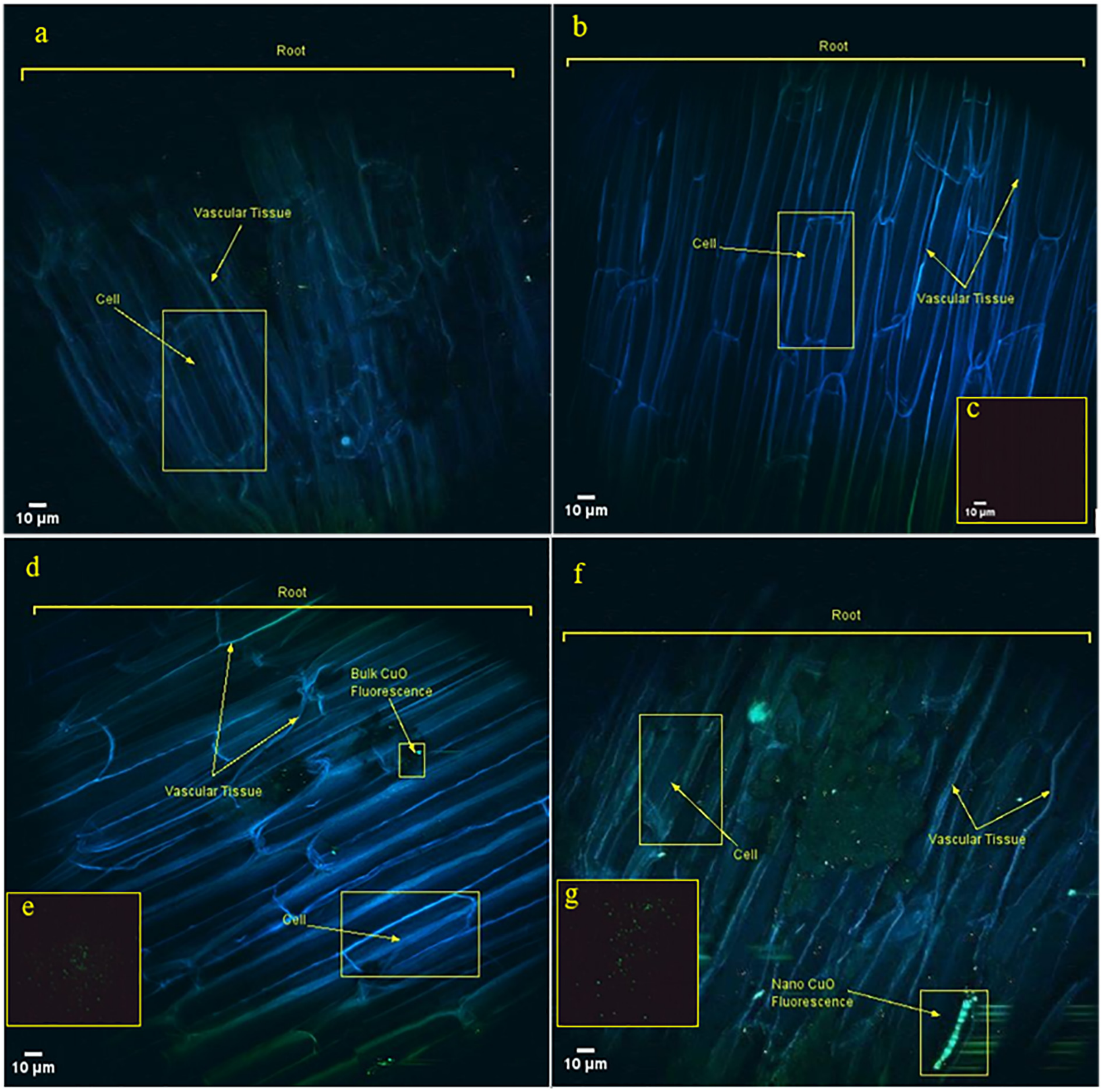

Fig. 1. Two-photon microscopy images of roots from control plants (a) and plants treated with (b) $CuSO_4$, (d) bCuO and (f) nCuO at 600 mg/kg. The inserted pictures correspond to (c) $CuSO_4$ in DI water, (e) pure bCuO suspension in DI and (g) pure nCuO suspension in DI. The fluorescence of root cell walls and vascular tissue is shown. Arrows inside the square indicate fluorescence from bCuO (d) or nCuO (f) particles.

In this study, the maximum Cu concentration in aerial parts of plants exposed to 75 mg/kg soil was lower than 32 mg/kg DW (Fig. 2b and c). Scallion is consumed in small amounts as a seasoning; thus, this Cu concentration, independently if it is present in nano, bulk, or ionic form, appears to be safe for human consumption (Bost et al., 2016). According to our two-photon studies performed in root tissues, Cu might be present in nanoforms. Hence, it can be expected to find nano Cu in leaves; nevertheless, further experiments are needed to confirm it. Similar results were reported by Soudek et al., who exposed *Allium* plants to 46.9 mg/L $Cu(NO_3)_2$ (Rastilantie et al., 2009). These authors found that Cu was mainly accumulated in roots (~5500 mg/kg DW) with small translocation to bulbs (~120 mg/kg DW) and leaves (~10 mg/kg DW).

3.3. Elements accumulation in tissues

Data on macro and microelements uptake showed that nCuO, bCuO, and $CuSO_4$, differentially affected the accumulation of essential elements (Ca, K, Mg, P, Fe, Mn, Ni) and Al (a non-essential element) in scallion tissues. Undisturbed essential elements have not been discussed in this work.

3.3.1. Macronutrient elements in roots

Fig. 3 shows the concentration of macroelements that were affected by Cu treatments. At the root level, there were significant changes in the accumulation of Ca, K, Mg, and P (Fig. 3a–d).

3.3.1.1. Calcium. Compared with control (16,607 mg/kg DW), only nCuO at 150 mg/kg (30,971 mg/kg DW) and at 300 mg/kg (32,621 mg/kg DW) induced a significant increment on root Ca content (Fig. 3a) ($p \leq 0.05$). Conversely, bCuO and $CuSO_4$ at 150 mg/kg, with values of ~5500 and ~6600 mg/kg, respectively, showed a significant reduction in root Ca when compared with control ($p \leq 0.05$). Excess Cu induces the overproduction of reactive oxygen species (ROS), which alter the permeability of Ca channels, interfering with Ca uptake by root cells (Demidchik et al., 2007). Additionally, bCuO particles are more negatively charged compared with nCuO (Table S1), which may result in a

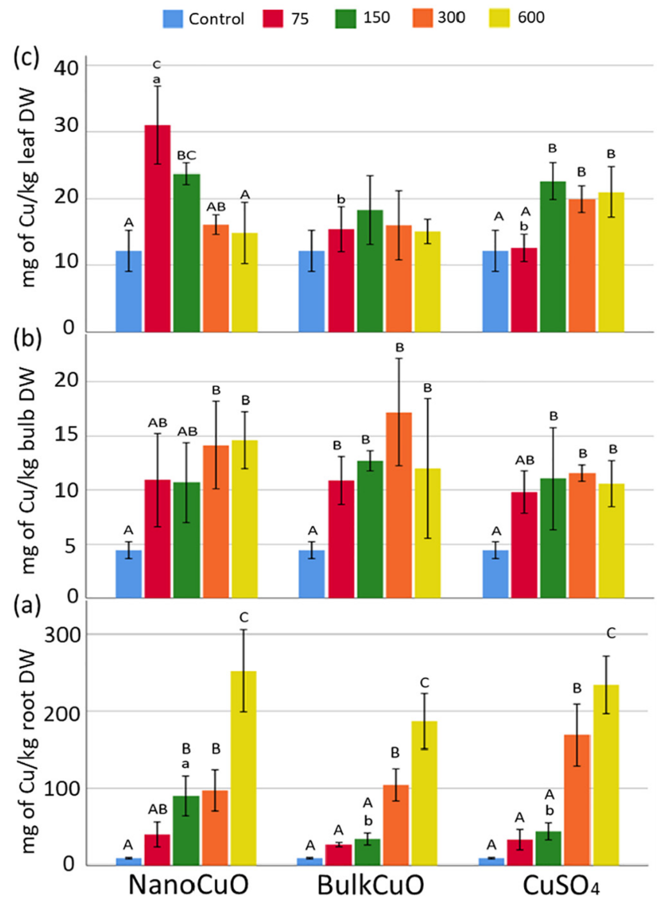

Fig. 2. Copper concentration (mg/kg DW) in scallion roots (a), bulbs (b) and leaves (c) exposed to nCuO, bCuO, and $CuSO_4$ at 0, 75, 150, 300 and 600 mg/L. Data are the average of three replicates ± standard error (SE). Lowercase letters stand for statistical differences among different compounds at the same concentration. Uppercase letters represent significant differences among different concentrations of the same compound ($p \leq 0.05$, n = 3).

higher attraction for positive Ca^{2+} ions at the root-soil interface. Consequently, a reduction in Ca^{2+} mobility should be expected and would explain, at least in part, the lower Ca uptake by root cells in plants treated with bCuO compounds. Regarding the lessening of Ca content caused by the ionic treatment, it has also been reported that ionic Cu^{2+} reduced Ca^{2+} concentration in Norway spruce (*Picea abies*) (Trujillo-Reyes et al., 2014). This species was treated with elevated concentrations of ionic Cu (1.5 μM), which resulted in a decreasing of Ca content in roots and wood. Moreover, Ca addition diminished the accumulation of Cu, indicating a competing relationship between Cu and Ca elements (Österås and Greger, 2006).

3.3.1.2. Potassium. None of the treatments altered K concentration, compared with control. However, K uptake was differentially affected by the diverse Cu compounds evaluated at the same concentration (Fig. 3b). For instance, at 75 and 150 mg/kg treatments, bCuO significantly enhanced root K (57,663 and 61,969 mg/kg DW, respectively) compared with nCuO (39,602 and 45,668 mg/kg DW, respectively) ($p \leq 0.05$). Interestingly, this was contrary to root Cu uptake, where nCuO at 150 mg/kg increased Cu content respecting to bCuO (Fig. 2a) ($p \leq 0.05$). Thus, higher root Cu acquisition generated by nCuO treatment reduced K content in scallion plants. Concurring with these findings, an increase in Cu^{2+} led to a decrease of K^{+} in *Arabidopsis* root cells (Demidchik et al., 1999). Regarding $CuSO_4$-exposed plants in the present study, no significant changes were registered, thus, the observed responses suggest this effect is driven by the Cu particles. In previous literature, Wang et al. reported that nCuO increased K^{+} leakage in maize (*Zea mays*) plants due to damages in the lipid bilayer (Wang et al., 2012).

3.3.1.3. Magnesium. Compared with control, bCuO, at all concentrations, except 300 mg/kg, significantly decreased root Mg up to 33% ($p \leq 0.05$) (Fig. 3c). The reduction in bCuO treatments were significantly more compared with nCuO. The pattern on Mg and Ca accumulation in plants exposed to nCuO and bCuO treatments was similar (Fig. 3a and c). This behavior may be explained since both Ca and Mg share the same transporter, namely AtCNGC10 (Guo et al., 2010), which suggests that nCuO may induce AtCNGC10 expression, facilitating Mg^{2+} transport inside cells. However, the latter needs a further experimental demonstration.

3.3.1.4. Phosphorus. $CuSO_4$ at 600 mg/kg significantly augmented P in roots, compared with nCuO (45.7%), bCuO (47.1%), and control (24.2%) (Fig. 3d) ($p \leq 0.05$). A possible explanation about the increment in P at higher $CuSO_4$ doses may be related to an up-regulation of the Pht1 transporter. Nevertheless, there exists the possibility that particles of nCuO or bCuO physically blocked the P transporters, reducing its uptake (Zuverza-Mena et al., 2015). Rawat et al. reported that bCuO at 500 mg/kg, reduced P absorption in bell pepper roots by 36%, compared with control (Rawat et al., 2018). Hong et al. found a reduction of root P in lettuce and alfalfa plants exposed to bCuO and nCuO at 5, 10, 20 mg/L (Hong et al., 2015). This later work also reported that 10 and 20 mg/L of $CuCl_2$ resulted in the highest P accumulation in alfalfa shoot ($p \leq 0.05$). None of the remaining treatments affected root P, compared with control.

3.3.2. Micronutrients and aluminum in roots

Fig. 3 also shows the concentration of the microelements that were affected by the Cu treatments in roots. There were changes in the accumulation of Fe, Mn, Al and Ni, this latter a non-essential element (Fig. 3e–h).

3.3.2.1. Iron. Fe in roots (Fig. 3e) was significantly increased by nCuO at 75 and 150 mg/kg (3070 and 3622 mg/kg), compared with bCuO (1165 and 1437 mg/kg), $CuSO_4$ (1496 and 1439 mg/kg), and control (1797 mg/kg) ($p \leq 0.05$). The observed differences between nCuO and $CuSO_4$ at ≤150 mg/kg suggest that Fe uptake was due to an effect of particle size and/or surface properties and ion release. Hence, Cu ions released from $CuSO_4$ may compete with Fe for transporters active sites, inhibiting its uptake (Apodaca et al., 2017; Hong et al., 2015). Additionally, Deák et al. suggested that the stress caused by nanoparticles increased the production of ferritin, an intracellular protein with a high affinity to bind Fe (Deák et al., 1999).

3.3.2.2. Manganese and aluminum. Fig. 3f shows that Cu treatments did not affect root Mn content, compared with control. However, there were differences among Cu forms evaluated at the same concentration. When the plants were exposed to 150 and 300 mg/kg of nCuO, bCuO, and $CuSO_4$, the roots treated with nCuO significantly showed higher Mn than bCuO and $CuSO_4$ ($p \leq 0.05$). Similarly, nCuO at 75–300 mg/kg, increased root Al content compared with bCuO and $CuSO_4$ (Fig. 3g) ($p \leq 0.5$). It is possible that the uptake of Mn and Fe is affected by similar mechanism; thus, the negativity of bCuO and the high amount of Cu ions released by $CuSO_4$ might be affecting Mn uptake in a similar manner than Fe uptake. An increase in Al uptake was previously reported in lettuce hydroponically exposed to Cu/CuO NPs (Trujillo-Reyes et al., 2014). The uptake of Al may follow a comparable trend than similar cations. However, because Al is not an essential element, not much discussion on its uptake is presented herein.

3.3.2.3. Nickel. Differences in Ni concentrations at the root level are presented in Fig. 3h. A significant reduction in Ni content was found when plants were exposed to 600 mg/kg of nCuO, 150 mg/kg of bCuO, and ≤150 mg/kg of $CuSO_4$, compared with control ($p \leq 0.05$). However, the

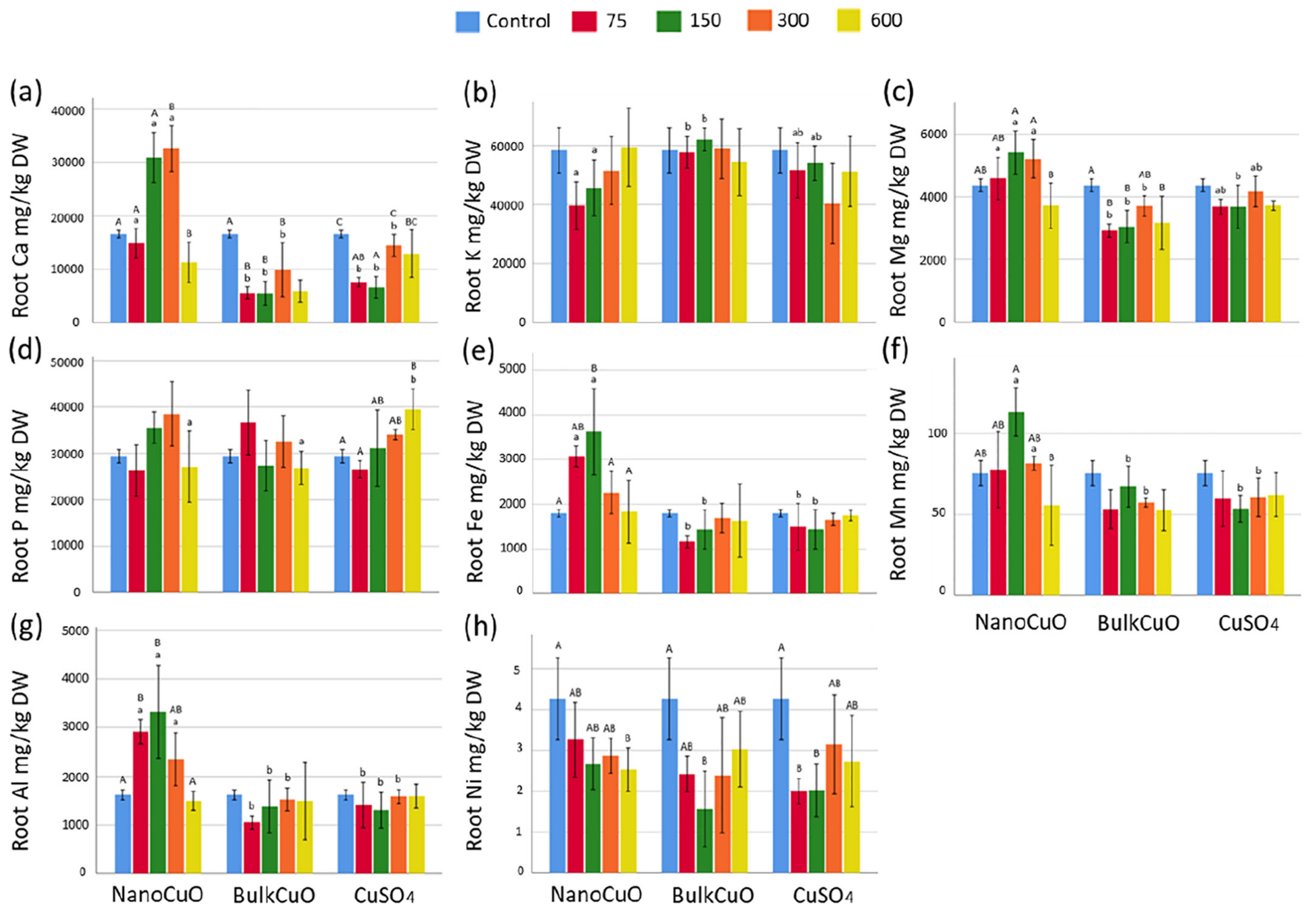

Fig. 3. Concentration (mg/kg DW) of the elements (a) Ca, (b) K, (c) Mg, (d) P, (e) Fe, (f) Mn, (g) Al, and (h) Ni, in scallion roots. Plants were cultivated for 80 days in soil amended with nCuO, bCuO, and $CuSO_4$ at 0 (control), 75, 150, 300 and 600 mg of Cu/kg of soil. Data are averages of three replicates ± standard error (SE). Lowercase letters stand for statistical differences among different compounds at the same concentration. Uppercase letters represent significant differences among different concentrations of the same compound ($p \leq 0.05$).

Ni content did not exhibit a particular trend with increasing doses of the Cu compounds. The literature has shown that Ni^{2+}, Cu^{2+}, and Zn^{2+} are taken up by the same carrier (Cataldo et al., 1978a, 1978b); thus, excess of Cu^{2+} may compete with Ni^{2+} uptake.

3.3.3. Essential elements in bulbs and leaves

Fig. 4 shows the concentration of macro and microelements that were affected by the Cu treatments in bulbs. Basically, lower Cu acquisition and lesser alteration were observed in bulbs than in roots, which suggested a "phytostabilization effect" of the underground tissues of scallion.

Plants exposed to nCuO at 150 mg/kg had significantly more Ca and Mg in bulbs, compared with control (74% and 108%) (Fig. 4a and b). Bulk CuO and $CuSO_4$ did not modify Ca and Mg accumulation in bulbs, compared to controls. The delivery of Mg to edible tissues is determined by the MRS2, MHX, and the MRS2-11 transporters (Li et al., 2017), which suggests that only nCuO, at 150 mg/kg, could have had an effect on their transport activity. The average Ca and Mg contents in scallion

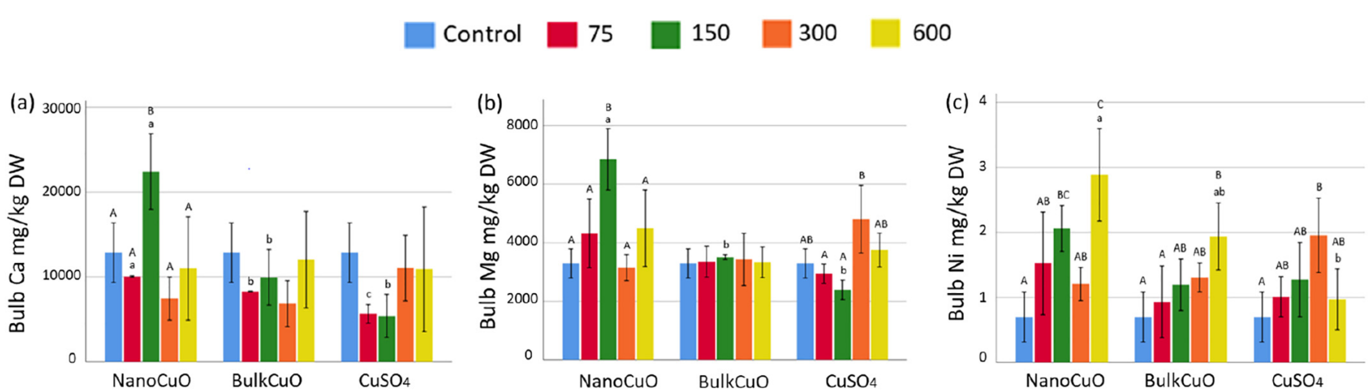

Fig. 4. Concentration (mg/kg DW) of the essential elements (a) Ca, (b) Mg, and (c) Ni in dry bulbs of scallion plants. Green onions were cultivated for 80 days in soil amended with nCuO, bCuO, and $CuSO_4$ at 0 (control), 75, 150, 300 and 600 mg of Cu/kg of soil. Data are averages of three replicates ± standard error (SE). Lowercase letters represent statistical differences among different compounds at the same concentration. Uppercase letters represent significant differences among different concentrations of the same Cu-based compounds ($p \leq 0.05$).

bulbs reported by the U.S. Department of Agriculture are 720 and 200 mg/kg fresh weight (~7200 and 2000 mg/kg DW) (Food Composition Databases Show Foods – Onions, spring or scallions (includes tops and bulb), raw, 2018), which are lower than the average values of control (12,846 and 3286 mg/kg DW). This may be due to the variety or the experimental conditions. The adequate intake of Mg and Ca for adult are ~300 mg/d and 1000 mg/d (Moore-Schiltz et al., 2015), respectively. The results of the current study revealed that nCuO improves the nutritional values of scallion bulbs by increasing Mg and Ca concentrations. In bulbs, Nickel showed an increase with rising doses of Cu compounds (Fig. 4c). Cataldo et al. stated that in soybean plants Ni^{2+} tends to form organic complexes within tissues, which are carried out through the xylem and deposited in the leaf and in the seeds of the soybean plants (Cataldo et al., 1978a). In bell pepper, Ni was found in fruit (Rawat et al., 2018). This suggests the distribution of Ni in plants is species dependent.

3.4. Plant growth and chlorophyll content

In this study, none of the Cu-based compounds caused significant changes in fresh weight, plant height, water content, and chlorophyll, compared with control (data are shown in Figs. S1 and S2, $p \leq 0.05$). No obvious signs of toxicity were found through the growth period. However, a comparison between treatments showed that nCuO treatments, except at 75 mg/kg, had significantly higher plant height, compared with bCuO and $CuSO_4$. This was probably due to the increase in nutrient elements given by nCuO, which benefits plant growth. Finally, the height of the plants treated with nCuO at 75 mg/kg and controls did not significantly vary, which suggests that "lower" concentrations of nCuO in soils do not pose implications for the green onion market value.

3.5. Allicin content

The effect of Cu-based treatments on leaf allicin content is depicted in Fig. 5. As shown in the figure, nCuO and $CuSO_4$ at all concentrations, significantly increased allicin content in the range 56–187% and ~34–90%, respectively, compared with control ($p \leq 0.05$). Meanwhile, bCuO treatments did not affect allicin content. This outcome was somehow in agreement with leaf Cu contents at the same treatments (Fig. 2c). Allicin has a beneficial effect in preventing alterations of lipid profile induced by Cu (Metwally and Metwally, 2009). In addition, allicin scavenges ·OH, which reduces lipid peroxidation (Prasad et al., 1995). Thus, it is possible that the increase in allicin concentration was due to counteracting the oxidative stress caused by the Cu-based treatments.

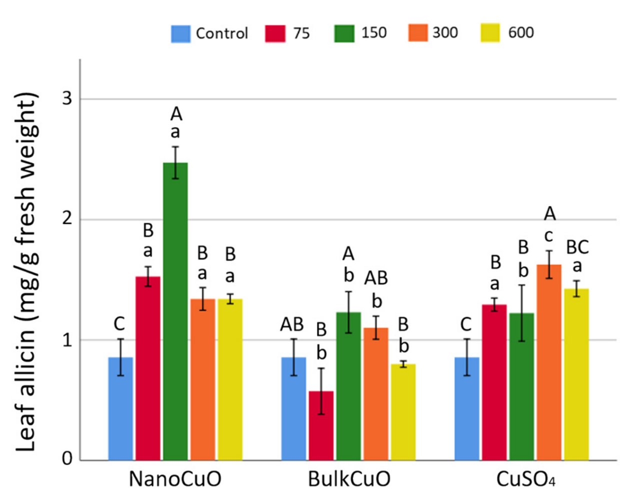

Fig. 5. Allicin content (w%) in leaves of plants cultivated for 80 days in soil amended with nCuO, bCuO, and $CuSO_4$ at 0, 75, 150, 300 and 600 mg/L. Data are averages of three replicates ± standard error (SE). Lowercase letters stand for significant differences among different compounds evaluated at the same concentration. Uppercase letters represent statistical differences among the same compound at different concentrations, compared to control (n = 3, $p \leq 0.05$).

3.6. Antioxidant defense system in roots and leaves

Fig. 6 shows the activity of APOX, CAT, GPOX, and SOD in different scallion tissues. In root tissue, the nCuO at 75–300 mg/kg increased GPOX by 121.5–245.7% (Fig. 6a), while at ≥150 mg/kg reduced CAT activity by 44.6–58.2% (Fig. 6c), respect to control ($p \leq 0.05$). These results suggest that nCuO modulates the enzyme antioxidant responses by compensating the activity of particular enzymes. Contrarily, none of the treatments affected APOX (Fig. 6b) and SOD (Fig. 6d) activities in the root. At leaf levels, no changes were observed in GPOX and SOD. However, APOX and CAT activities in leaves presented some alterations, as shown in Fig. 6e and f, respectively. Plants exposed to bCuO at all concentrations, and nCuO and $CuSO_4$ at concentrations ≥150 mg/kg, showed a significant APOX activity increment in leaves (Fig. 6e) ($p \leq 0.05$). Finally, nCuO at all concentrations significantly reduced leaf CAT activity (Fig. 6f), compared with control ($p \leq 0.05$). In this study, the activity of stress-related enzymes from roots and leaves were differentially affected by Cu compounds. For instance, the nCuO significantly induced GPOX (Fig. 6a) activity and reduced CAT (Fig. 6c) in roots, while the three Cu-based treatments augmented APOX activity in leaves (Fig. 6e). These results suggest a specific size effect of nCuO, compared with the other two compounds. Also, it seems that nCuO treatments induce higher oxidative stress, although further ROS measurements are required to confirm this. It can be speculated that the nCuO interacts with the binding sites of CAT reducing its activity, while APOX-increased activity in leaves may be a direct response to reduce the stress imposed by the three compounds (Rico et al., 2013; Stark, 2011).

The forthcoming formation of peroxy radicals in roots induced by nCuO enhanced root GPOX (Fig. 6a), while in leaves the increased APOX activity (Fig. 6e) may be caused by high H_2O_2 formation. Since the APOX-H_2O_2 affinity is higher than CAT-H_2O_2 (Lin et al., 2009). the increased activity of APOX in leaves was possibly enough to reduce the oxidative stress; then, no further increase of CAT was needed in the leaves. Additionally, to support the enzymatic antioxidant defenses, the data suggest that the plants produced a higher allicin content to combat an excess of ·OH in leaves.

4. Conclusions

This study describes the physiological responses of scallion plants exposed to nano, bulk, and ionic Cu-based compounds. Two-photon microscopy showed that both nano and bulk CuO compounds were taken up by the roots, and none of the Cu-based tested concentrations produced visible signs of toxicity. Though, significant physiological and biochemical changes were observed in mature plants. Scallion plants exposed to nCuO at 75–300 mg/kg, improved their nutrient quality by increasing the allicin and essential element (Ca, Fe, Mg, Mn, and Ni) contents. This was, in most of the cases, contrary to the results observed with bCuO. Nano CuO significantly increased root GPOX and leaf APOX, and reduced CAT in both roots and leaves. This pattern on enzyme activities suggests that nCuO compounds induce higher oxidative stress, although more experiments are needed to confirm it. Overall, the data demonstrated that nCuO improved the allicin, Ca, Fe, Mg, and Mn contents and did not impair the agronomical parameters. Finally, more studies on safety issues are required to consider the use of nCuO as potential nanofertilizer.

CRediT authorship contribution statement

Yi Wang: Conceptualization, Investigation, Writing - original draft. **Chaoyi Deng:** Conceptualization, Investigation, Writing - original draft. **Keni Cota-Ruiz:** Visualization, Supervision, Writing - review &

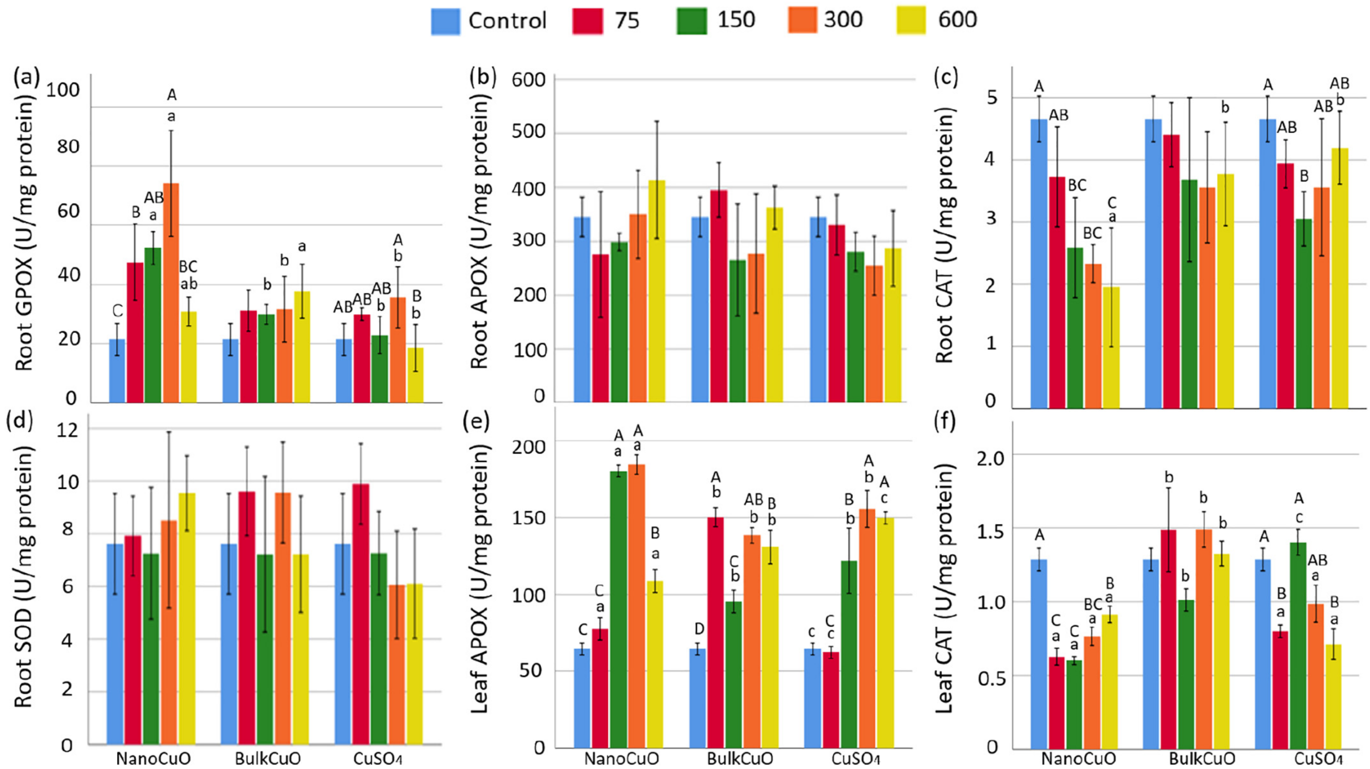

Fig. 6. Enzyme activities of CAT, GPOX, SOD, and APOX in the roots and leaves of soil-grown green onion plants. Chinese scallions were exposed to nCuO, bCuO, and $CuSO_4$ at 0, 75, 150, 300 and 600 mg/L. Data are the average of three replicates ± standard error (SE). Lower letters represent statistical differences among different compounds tested at the same concentration. Uppercase letters stands for statistical differences of the particular compound tested at different levels, compared to control (n = 3, $p \leq 0.05$).

editing.**Jose R. Peralta-Videa:** Conceptualization, Investigation, Visualization, Supervision, Writing - review & editing. **Youping Sun:** Investigation, Visualization, Supervision. **Swati Rawat:** Investigation. **Wenjuan Tan:** Investigation. **Andres Reyes:** Investigation. **Jose A. Hernandez-Viezcas:** Investigation, Visualization, Supervision. **Genhua Niu:** Conceptualization, Investigation, Visualization, Supervision. **Chunqiang Li:** Investigation, Visualization, Supervision. **Jorge L. Gardea-Torresdey:** Conceptualization, Investigation, Visualization, Supervision, Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.138387.

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