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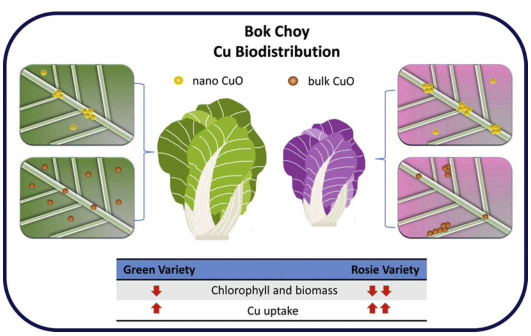
Bok choy (*Brassica rapa*) grown in copper oxide nanoparticles-amended soils exhibits toxicity in a phenotype-dependent manner: Translocation, biodistribution and nutritional disturbance

Chaoyi Deng[a,c,1], Yi Wang[b,c,1], Keni Cota-Ruiz[b,c], Andres Reyes[d], Youping Sun[e], Jose Peralta-Videa[a,b,c], Jose Angel Hernandez-Viezcas[b,c], Reagan S. Turley[b,c], Genhua Niu[e], Chunqiang Li[d], Jorge Gardea-Torresdey[a,b,c,*]

[a] *Environmental Science and Engineering Ph.D. Program, The University of Texas at El Paso, 500 West Univ. Ave., El Paso, TX 79968, USA*
[b] *Department of Chemistry and Biochemistry, The University of Texas at El Paso, 500 West Univ. Ave., El Paso, TX 79968, USA*
[c] *University of California Center for Environmental Implications of Nanotechnology (UC CEIN), The University of Texas at El Paso, 500 West Univ. Ave., El Paso, TX 79968, USA*
[d] *Department of Physics, The University of Texas at El Paso, 500 West Univ. Ave., El Paso, TX 79968, USA*
[e] *Texas A&M Agrilife Research and Extension Centre at El Paso, 1380 A&M Circle, El Paso, USA*

GRAPHICAL ABSTRACT



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ABSTRACT

The comparative toxicity of nano/bulk cupric oxide (CuO) and ionic copper (Cu) in Rosie and Green bok choy (*Brassica rapa*) varieties, with higher and lower anthocyanin contents, respectively, was investigated. Both phenotypes were cultivated for 70 days in natural soil amended with nano CuO (nCuO), bulk CuO (bCuO), and Cu chloride ($CuCl_2$) at 75, 150, 300, and 600 mg Cu/kg soil. Essential elements in tissues, agronomical parameters, chlorophyll content, and Cu distribution in leaf were determined. In both varieties, nCuO treatments significantly increased Cu uptake in roots, compared with bCuO and $CuCl_2$ ($p \leq 0.05$). At all treatment concentrations, Rosie variety had more Cu than Green. More physiological impairments such as chlorophyll and leaf biomass reduction were observed in treated-Rosie varieties, compared to Green plants. The adverse effects were higher in nCuO-treated plants than their bCuO- or ionic Cu-exposed counterparts. Different distribution patterns of the translocated Cu in leaf midrib and parenchyma depended on particle size and plant phenotype, as demonstrated by two-photon microscopy. The different effects of CuO-based compounds in Rosie and Green

* Corresponding author at: Department of Chemistry and Biochemistry, The University of Texas at El Paso, 500 West University Avenue, El Paso, TX 79968, USA.
E-mail address: jgardea@utep.edu (J. Gardea-Torresdey).
[1] These authors contributed equally.

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varieties may be related to the anthocyanin content. These findings help to understand the factors involved in nanoparticles uptake and translocation to plant edible parts.

1. Introduction

Nanotechnology has become one of the most promising and fastest-growing industries of the 21st century. It is estimated that by 2022 the global market for nanoparticles (NPs) may surpass 7.3 billion dollars (McWilliams Andrew, 2018). By 2010, the manufacturing of copper oxide (CuO) NPs, one of the most extensively applied Cu-based materials, was higher than 200 metric tons per year (Keller et al., 2013). CuO-based NPs have been widely utilized in batteries, lubricants polymers, pigments, gas sensors, and catalysts, among others (Anjum et al., 2015).

Additionally, the production and use of CuO NPs in the agrosystem is increasing (European Commission, 2018). They are showing the potential to be applied as nanofungicides, nanopesticides, and nanoherbicides (Elemike et al., 2019; Pestovsky and Martínez-Antonio, 2017). Plants are an essential base in the ecological environment and are being exposed to NPs. Hence, they may play a critical role in the transport and bioaccumulation of NPs (Abdallah et al., 2020). When the NPs accumulate in the edible parts at a high level, it may pose a risk to human health.

Bok choy (*Brassica rapa* subsp. *chinensis*) vegetables are well-known for their sweet flavor, high protein, vitamin, mineral content (Rochfort et al., 2006), and also for their use as a potential agent against cancer (Wang et al., 2015). There are two popular varieties: Green with green leaves and Rosie with purple leaves. The principal difference between these varieties is the content of anthocyanin pigments. Rosie leaves contain more than fifteen types of anthocyanins, totaling ~3 mg/g dry weight (DW), while Green leaves have nearly no anthocyanin (Zhang et al., 2014). Anthocyanins are water-soluble flavonoids pigments which are responsible for developing the characteristic red, purple, and blue colors, among others, in numerous plants. According to previous studies, anthocyanins are considered healthy for human beings because they are antioxidants and have anti-inflammatory properties. Furthermore, anthocyanins chelate trace metals and are involved in lipid peroxidation inhibition processes (He et al., 2016). They may also attenuate damage from cold, drought, and UV irradiation in plants (Hosseinian and Beta, 2007).

Our knowledge about the impacts of CuO nanoparticles (nCuO) on edible vegetables, especially cultivated in nature soils and under long-term exposure, is still limited. Some studies have shown that nCuO elicits both positive and negative physiological effects in plants. These responses include changes in the content of macro/micronutrients, sugar, protein, as well as modifications in root length and plant biomass (Abdel-Wahab et al., 2019; Gao et al., 2018; Nalci et al., 2019; Spielman-Sun et al., 2018). The toxic effects of CuO NPs have been reported in several species. For example, CuO NPs shortened root length and affected its morphology in barley (*Hordeum sativum* distichum) (Rajput et al., 2018), inhibited wheat growth (*Triticum aestivum*) (Gao et al., 2018), and impaired the nutritional quality of bell pepper (*Capsicum annum* L.) plants (Rawat et al., 2018). Remarkably, a few studies have shown that the anthocyanin content may be involved in plants' physiological and biochemical behavior in response to NPs exposure. For instance, Tan et al. (2018) foliarly-applied $Cu(OH)_2$ nanowires at 4.8 mg Cu/per pot onto two basil (*Ocimum basilicum*) varieties, with different anthocyanin content; the investigation reported variety-dependent Cu accumulation and fatty acid profile alterations. Thus, it triggered our interest to study the plant variety-dependent responses to nCuO exposure.

Two-photon microscopy is receiving considerable attention due to its deeper tissue penetration and its strong signal background suppression (Helmchen and Denk, 2005). It has the advantage of viewing the internal structure via fluorescence without the need for extensive sample preparation (Bonilla-Bird et al., 2018). This technique was applied in this study to analyze the translocation and biodistribution pattern of nano and bulk CuO particles in Green and Rosie leaves.

There is currently no literature about the effect of nCuO on bok choy plants. Moreover, how different phenotype traits affect the translocation and biodistribution of NPs in plant tissues is still unknown. Therefore, we aimed to characterize the physiological responses of two bok choy varieties treated with nCuO, bCuO and ionic Cu. To understand these responses, we measured Cu uptake and biodistribution, chlorophyll content, biomass production, nutrient elements accumulation, and sugar and starch content in bok choy tissues.

2. Materials and methods

2.1. Potting soil preparation

Rounded plastic pots (25 cm × 26 cm) were washed with Milli-Q 18 MΩ deionized water (MW) and filled with 5 kg of natural soil amended with the respective Cu compound/concentration (see below). The soil was collected from Socorro, TX, USA (latitude: 31°67′ N and longitude: 106°28′ W, elevation: 1115 m asl) and its physicochemical properties are shown in Supplementary file.

2.2. Preparation of nanoparticle suspensions and other treatments

Particulate nCuO, bCuO, and ionic Cu ($CuCl_2$) compounds were obtained from The University of California Center for Environmental Implications of Nanotechnology (UC-CEIN). Their physicochemical properties have been previously reported and are presented in Table S1 (Hong et al., 2015; Rawat et al., 2018). Suspensions/solutions of the Cu-based products were prepared in MW and applied to the soil to reach final Cu concentrations of 75, 150, 300, and 600 mg/kg of soil. These concentrations were selected due to previous reports which suggested Cu concentrations in soils can be found in the ranges mentioned above (Kulikowska et al., 2015; Yang et al., 2017a,b). Suspensions/solutions were freshly prepared and sonicated (Crest Ultrasonic, Trenton, NJ) in a water bath at 20 − 25 °C for 25 min and 180 W. Bulk and nCuO were used to compare the effect of particle size, while the ionic compound was used to differentiate the effects of particles and ions. After sonication, the corresponding suspension/solution was added to 5 kg of soil and manually mixed for 30 min until homogeneity was achieved. Control plants were only treated with MW. Soil moisture was maintained at near 60 % of field capacity. Besides, ζ-potential of 300 mg/L nCuO and bCuO suspensions with a pH range from 3 to 10 was measured by a Malvern Zetasizer Nano ZS-90 following a laser deppler electrophoresis procedure.

2.3. Seed germination and plant growth

Green and Rosie (F1, Hybrid) bok choy seeds (*Brassica rapa* var. *chinensis*) were purchased from Johnny's Selected Seeds (Winslow, ME). Seeds were washed and submerged in 2 % hypochlorite (NaClO) solution to avoid contamination, rinsed with MW three times, placed in a 250 mL Erlenmeyer flask with 200 mL of MW, and stirred for 12 h for hydration. Then, two seeds were planted equidistantly in germination trays containing potting mix (Miracle Grow®) at 1 cm deep and grown in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) at photosynthetic photon flux density (PPFD) of 50 μmole m^{-2}

s^{-1},14 h photoperiod, 25/20 °C day/night temperature, and 65–70 % relative humidity. Seedlings were watered with 10 mL of MW per day. Three-week-old seedlings were transplanted into new pots (1 seedling per pot) containing the Cu-amended soils at the conditions mentioned above and placed in a greenhouse at 30/20 °C average day/night temperature, 50–75 % relative humidity and light daily integral of ~10 mol m^{-2} d^{-1}. Each pot was irrigated with a nutrient solution, which was made by adding water-soluble fertilizer (N 15 %, K_2O 15 %, P_2O_5 5%) to reverse osmosis water. Plants were harvested 50 days after transplanting.

A factorial design was arranged in a completely random way. The main factors were the varieties (Rosie, Green) and Cu-based compounds (nCuO, bCuO, $CuCl_2$) at five treatment concentrations (0, 75, 150, 300, and 600 mg/kg). Pots without Cu application were considered as controls.

2.4. Physiological and biochemical parameters

Before harvesting, the foliar leaf area was measured using an area meter (LI-3100, LI-COR, Lincoln, NE). After harvesting, chlorophyll content was evaluated. Briefly, 0.5 g of fresh leaf samples were ground, and the chlorophyll was extracted with 80 % acetone. The absorbances were measured as described by Porra (2002). To determine mass of collected tissues, DW was recorded after oven-dried tissues for 3 d at 72 °C. Leaf pH value was evaluated with the Malvern Zetasizer Nano ZS-90 instrument. For sugar and starch determination, dry leaves (100 mg) were weighed and analyzed according to Dubois et al. as described in SI (DuBois et al., 1956).

2.5. Macro and microelement determination

Oven-dried and digested tissue samples were analyzed for macro and microelements by inductively coupled plasma–optical emission spectrometry (ICP-OES, Perkin-Elmer Optima 4300 DV; Shelton, CT). The detailed method is described in the supporting information (SI).

2.6. Sample preparation for Two-photon microscopy images

Leaf samples of both Rosie and Green plants exposed to nCuO, bCuO, and $CuCl_2$ at 600 mg/kg, were harvested at day 50, dried and mounted in water-immersion objective lens (Olympus LUM Plan FLN) (Bonilla-Bird et al., 2018). The light source used in the microscope was a mode-locked femtosecond Ti: Sapphire laser (Spectra-Physics, Mai-Tai HP). The pulse duration was ~100 fs width, and the repetition rate was 80 MHz. To achieve two-photon excitation, the wavelength was set at 710 nm light, and 200 mW laser power was delivered at the sample site. The fluorescence signal from the sample was deflected with a 665 nm long-pass dichroic mirror. A long-pass dichroic beam splitter (LDBS) was used to split the blue and green/red fluorescence signal. Blue, green, and red signals were transmitted through band-pass filters of 417 − 477 nm, 500 − 550 nm, and 570 − 616 nm, respectively, and finally, detected by a Photo Multiplier Tube (PMT) respectively. The outputs of these three PMTs were fed into red/green/blue channels of a frame grabber installed on a computer. Two-dimensional images in the x–y plane were acquired through a custom software program. The imaging speed was 30 frames/sec and each final static image had an average of 50 frames. Furthermore, some images exhibited red artifacts, such as streaks across the image, when processed by the frame grabber; thus, the artifacts were removed through minimal image processing using the ImageJ 1.51j8 software (National Institute of Health, USA) (Acosta et al., 2014).

2.7. Statistical analysis

All the data were analyzed with the SPSS program 25 (SPSS, Chicago, IL, USA). To determine the multiple interaction effects of plant variety and compounds, a two-way ANOVA was used. Statistical significance was accepted at a p-value of 0.05. Pairwise comparisons were applied with a Bonferroni adjustment to compare differences between two treatments with a single main effect (plant variety, compound, or concentration). Reported data are means ± standard errors (SE) of triplicates. Pearson correlation coefficient analysis and one-way ANOVA followed by the Tukey–Kramer Post Hoc test were also used.

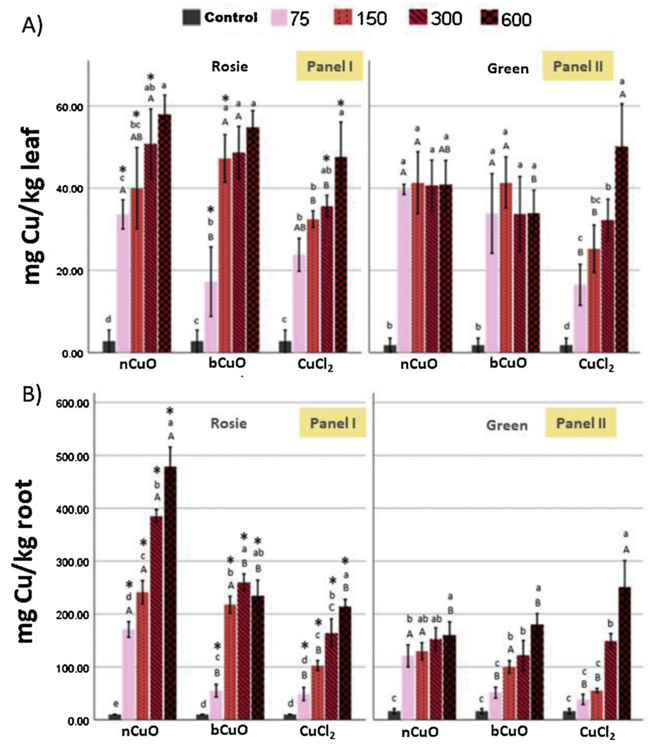

Fig. 1. mg of Cu/kg dry sample in (A) leaves, (B) root of both bok choy plants (70-day-old) cultivated in soil spiked with 0, 75, 150, 300, and 600 mg/kg of nCuO, bCuO, and $CuCl_2$. Data are means of three biological replicates ± Standard Error (SE). Capital letters stand for statistical differences between different compounds at the same concentration. Lowercase letters represent significant differences among the same compounds evaluated at different concentrations. Significant differences between the two varieties are marked with "*" ($p \leq 0.05$, n = 3).

3. Results and discussion

3.1. Cu is accumulated in roots and leaves in a variety-dependent manner

Results of Cu content in Rosie and Green bok choy varieties are shown in Fig. 1. Both varieties showed significantly higher Cu amounts in leaves (Fig. 1A) when they were treated with nCuO, bCuO, and $CuCl_2$ at all concentrations, compared with control ($p \leq 0.05$). In roots, nCuO and bCuO augmented Cu content in bok choy varieties at almost all evaluated concentrations, while $CuCl_2$ caused a variety-dose Cu accumulation response (Fig. 1B). Pearson correlation analysis (Table S4) demonstrated a significant positive relationship between root Cu content and treatment concentration in Rosie (0.74) and Green (0.86) cultivars ($p \leq 0.01$). The highest Cu concentration was found in Rosie plants treated with 600 mg/kg of nCuO as their roots contained up to ~479 mg Cu/kg DW ($p \leq 0.05$).

In this study, a variety-dependent Cu accumulation was found at root and leaf levels. Accordingly, Rosie varieties presented predominantly higher Cu amounts in their roots compared to Green variety in response to Cu exposure (Fig. 1B). Similarly, Rosie varieties treated with ≥300 mg/kg of nCuO or bCuO accumulated more Cu in their leaves than Green plants (Fig. 1A). As previously stated, Rosie plants have a higher content of anthocyanin compared to their Green counterparts (Zhang et al., 2014). Hence, a possible mechanistic explanation of higher accumulation of Cu in Rosie varieties may be related to the evidence that hydroxyl moieties of C ring from anthocyanin molecules chelate Cu^{2+} (Cortez et al., 2017; Welch et al., 2008). This is also supported by previous experimental and theoretical evidence which demonstrates that the *o*-di-hydroxyl groups from the B ring of anthocyanins can form anthocyanin-metal complexes (Boulton, 2001; Dai et al., 2012). Moreover, the O—H group, might inhibit the Fenton reaction process reducing the production of the harmful hydroxyl radicals (Primikyri et al., 2015).

Rosie roots treated with nCuO particles exhibited the highest Cu accumulation (Fig. 1B, Panel I). Possibly, nCuO particles, which possess smaller sizes, were easily associated to root plants and eventually uptaken. Additionally, a possible faster Cu ions release from nCuO may lead to the increase of Cu in Rosie root. However, these latter statements need a further experimental demonstration. Comparable findings were reported by Rawat et al. as they found higher root uptake in *Capsicum annum* plants exposed to nCuO (196 % compared with control) compared with ionic Cu compounds (184 % compared with control) (Rawat et al., 2018). Trujillo-Reyes et al. (2014) examined lettuce treated with different Cu compounds at concentrations of 0, 10, and 20 mg/L for 15 days. Results from this hydroponic study showed that lettuce plants accumulated more Cu from NPs than from ionic Cu treatments.

The translocation ratio between root Cu and leaf Cu was calculated by leaf Cu/root Cu content (Fig. S1). Interestingly, a lower translocation ratio was found in Rosie leaves (Fig. S1, Panel I) compared to Green cultivars (Fig. S1, Panel II). These results may be partly explained due to Cu excess/saturation in leaves from Rosie root plants (Fig. 1A, Panel I), compared with leaves from Green cultivars (Fig. 1A, Panel II). Additionally, this study showed that higher concentrations of Cu, in response to Cu-based treatments, were found in Rosie roots (Fig. 1B, Panel I). While it is true that particulate Cu compounds can be translocated from roots to shoots via the xylem (Dimkpa et al., 2013; Wang et al., 2012), their high density renders them sparingly mobile, inducing these compounds to remain at the roots (DalCorso et al., 2014). It may also contribute to understanding, to some extent, the lower translocation ratio observed in Rosie cultivars.

3.2. Cu is differentially distributed in leaves from plants exposed to nCuO and bCuO

Translocation of Cu to leaf tissues in plants treated with Cu from nCuO and bCuO was proved by two-photon microscopy (Fig. 2). Cu was found in leaves of both Rosie and Green varieties when plants were treated with 600 mg/kg of nCuO or bCuO. To guarantee that the fluorescent signal was emitted from Cu, control samples were used, and specific detection parameters for these compounds were previously set (Fig. S2). In Fig. 2, representative fluorescent spots of Cu are pointed out by arrows. They delineate that Cu signals from nCuO and bCuO exposure were distributed differently in leaf tissues. Remarkably, plants exposed to 600 mg/kg of nCuO or bCuO compounds showed different leaf Cu contents, as revealed by ICP-OES (Fig. 1A).

As shown in Fig. 2A, plant cells produced auto-fluorescence. However, the signal emitted from plants treated with nCuO was distinguishable, as indicated by arrows. Compared with Green cultivar (Fig. 2E), more fluorescent points were observed in Rosie varieties, which was consistent with the Cu uptake data obtained by ICP-OES (Fig. 1A). Cu fluorescence from nCuO was observed in leaf mesophyll/parenchyma (Fig. 2A, C) and midrib/vasculature (Fig. 2B, D). A higher intensity fluorescence and more signal points from plants treated with nCuO were found in midrib than in parenchyma in both varieties. This suggests that the vascular xylem tissue might mediate the translocation pathway from roots to leaves.

On the other hand, Cu resulting from bCuO exposure was more distributed in the lamina (Fig. 2E–H), compared with nCuO-treated plants (Lv et al., 2018). According to Supplementary Table 1, nCuO compounds possess smaller particle size and a less negative zeta potential (Fig. S3), compared with their bCuO counterparts (approximated values of −10 mV and −40 mV for nCuO and bCuO, respectively, at pH ~5.5), which might facilitate a possible nCuO aggregation in vein, preventing its further translocation to lamina. However, the aggregation of Cu as particulate compound needs further experimental demonstration. Additionally, different lamina translocation patterns were found between the two varieties (Fig. 2E and G). This phenomenon correlated with the leaves' pH and zeta potential values. Rosie leaves from control plants presented significantly lower pH values (5.51 ± 0.05), compared to the corresponding Green controls (5.76 ± 0.1) ($p \leq 0.05$) (Table S2). Although the difference is slight, it may affect the Cu content. No significant leaf pH changes were registered after exposing Rosie and Green plants to the different Cu

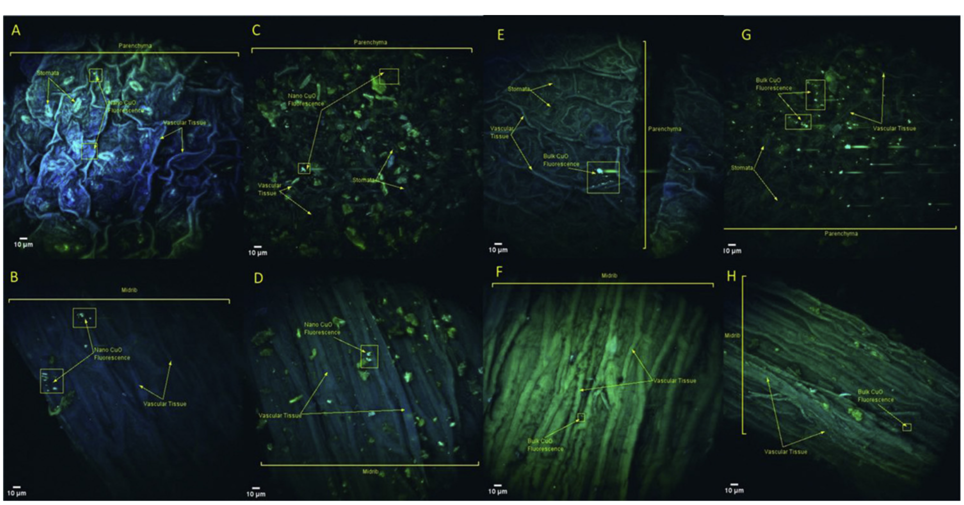

Fig. 2. Two-photon microscopy image of Rosie and Green varieties exposed to nCuO and bCuO at 600 mg/kg. (A) Rosie leaves parenchyma, exhibiting fluorescence from nCuO exposure as pointed out by arrows. Stomata and vascular tissues are also shown. (B) Rosie leaves midrib showing fluorescence from nCuO exposure which are pointed out by arrows. Vascular tissues are also labeled. (C) The parenchyma of Green leaves displaying fluorescence. (D) Leaves midrib of Green with Cu fluorescence from nCuO treatment, which is pointed out by arrows. The other arrows pointed out the structure of vascular tissues. (E) Leaves parenchyma of Rosie leaves showing Cu fluorescence from bCuO treatment as indicated by arrows in the square. The other arrows highlight the structure of stomata and vascular tissues. (F) Rosie midrib leaves exposed to bCuO. Cu fluorescence from bCuO exposure is pointed out by arrows (within the square). The other arrows specify the structure of vascular tissues. (G) Leaves parenchyma of Green plants exposed to bCuO. Fluorescence is pointed out by arrows. The other arrows describe the structure of stomata and vascular tissues. (H) Leaves midrib of Green plants exposed to bCuO. Cu fluorescence of plants grown with bCuO is shown in the square. The other arrows pointed out the structure of vascular tissues. All the images were obtained at 710 nm and 200 mW.

Table 1
Element content in bok choy varieties exposed to Cu-based compounds. Only significant changes compared to control are considered ($p \leq 0.05$). Variations are expressed in percentages.

Variety	Element	Part	Treatment (mg/kg soil)	Concentration (mg/kg DW)	%
Rosie	K	Leaf	Control	60717.747 ± 4591.298	100
			nCuO 150	33220.546 ± 6957.357	−45
		Root	Control	4045.079 ± 387.442	100
			nCuO 600	5995.870 ± 473.702	48
	P	Root	Control	2006.455 ± 109.530	100
			nCuO 600	1246.785 ± 136.012	−38
	Fe	Root	Control	8472.111 ± 609.394	100
			bCuO 75	5972.011 ± 507.181	−30
			CuCl2 75	6918.397 ± 142.921	−18
			CuCl2 600	4196.145 ± 259.856	−50
	Zn	Leaf	Control	33.071 ± 1.209	100
			nCuO 150	26.069 ± 1.156	−21
			nCuO 300	26.083 ± 1.937	−21
			bCuO 150	24.359 ± 1.197	−26
			bCuO 600	22.810 ± 0.913	−31
			CuCl2 150	25.569 ± 0.850	−22
			CuCl2 600	26.122 ± 1.460	−21
Green	K	Root	Control	5384.469 ± 112.333	100
			nCuO 75	4040.353 ± 346.190	−25
			nCuO 150	3710.340 ± 285.290	−31
			nCuO 600	3190.774 ± 93.624	−40
	P	Root	Control	3252.583 ± 384.356	100
			nCuO 150	980.040 ± 110.471	−70
			nCuO 600	1727.270 ± 422.065	−47
			bCuO 75	1591.778 ± 314.853	−51
			bCuO 150	1848.454 ± 21.090	−43
	Fe	Root	Control	7459.756 ± 376.851	100
			nCuO 150	5665.089 ± 319.718	−24
			nCuO 300	5132.094 ± 40.411	−31
			nCuO 600	5581.094 ± 348.802	−25
			bCuO 600	5043.778 ± 162.789	−32
			CuCl2 150	5356.109 ± 238.045	−28
			CuCl2 600	5344.325 ± 305.577	−28
	Zn	Leaf	Control	35.317 ± 1.151	100
			nCuO 300	27.350 ± 0.196	−23
			nCuO 600	26.550 ± 0.290	−25
			bCuO 600	22.327 ± 1.616	−37
			CuCl2 300	27.785 ± 2.275	−21
			CuCl2 600	27.320 ± 0.919	−23

compounds, compared to the corresponding controls ($p \leq 0.05$).

The mechanism of the uptake process of NPs by plants is still not well understood. Endocytosis is currently regarded as an uptake pathway of extracellular materials as well as NPs. In a previous study, it was observed that translocation of NPs from roots to shoots, independent of the root Ce concentrations and supplied forms of Ce (nano, bulk CeO or ionic Ce), occurred likely via endocytosis mechanism (Yang et al., 2017a,b). However, according to the results provided in this study, the whole process could be more complex. The concentration of NPs translocated upwards depended on not only endocytosis, but also the aggregation property of NPs in leaves.

3.3. Chlorophyll content decline is connected to anthocyanin-plant phenotype

The chlorophyll content in leaves at different treatment exposure is shown in Fig. 3. Chlorophyll-*a* and -*b* content were more decreased in Rosie than Green variety in both nCuO or bCuO treatments, compared with their respective controls ($p \leq 0.05$). In Rosie, fewer impairments on this parameter were caused by ionic compounds, which may be interpreted by their lower leaves Cu content (Fig. 1A).

A maximum fall in chlorophyll-*a* content was observed in Rosie plants treated with nCuO at 600 mg/kg with a reduction of ∼69 % (Fig. 3A, Panel I). In Rosie cultivars, the decrease in chlorophyll caused by nCuO was concentration-dependent; the higher the treatment concentrations, the lower the chlorophyll-*a* content.

In Green plants, nCuO and bCuO compounds significantly decreased leaves chlorophyll-*a* content at ≥150 mg/kg (Fig. 3A, Panel II) ($p \leq 0.05$). Nevertheless, patterns were different in comparison with Rosie variety since no concentration-dependent results were found. Meanwhile, $CuCl_2$ significantly reduced chlorophyll at all tested concentrations, compared with the control (Fig. 3A, Panel II).

As shown in Table S4, Pearson correlation analysis between leaves chlorophyll-*a* content and Cu concentration in root and leaves was −0.809 and −0.767 in Rosie, and −0.563 and −0.605 in Green variety, respectively ($p \leq 0.01$). These data showed evidence that chlorophyll-*a* was more reduced in Rosie plants than Green cultivars when the Cu concentration in soil was increased.

As previously shown, most impairments on chlorophyll-*a* concentrations were found in Rosie plants treated with particulate compounds compared to their Green counterparts. Interestingly, Rosie plants exhibited higher leaf Cu amounts when exposed to nCuO and bCuO (Fig. 1A, Panel I). This suggests that chlorophyll-*a* accumulation may be modulated by the interaction of anthocyanin with Cu from nCuO and bCuO exposure. However, more studies are needed to understand the interactive mechanisms among chlorophylls, Cu compounds, and anthocyanins.

Chlorophyll-*b* content in leaves is shown in Fig. 3B. In Rosie plants, nCuO and bCuO decreased chlorophyll-*b* content at ≥150 mg/kg, compared with control (Fig. 3B, Panel I) ($p \leq 0.05$). A significant reduction caused by $CuCl_2$ only occurred at 75 mg/kg, compared to control. Meanwhile, nCuO significantly reduced chlorophyll-*b* compared to $CuCl_2$ at ≥300 mg/kg. For the Green variety, none of the treatments caused significant different responses in chlorophyll-*b* compared with control (Fig. 3B, Panel II) ($p \leq 0.05$). Similar to

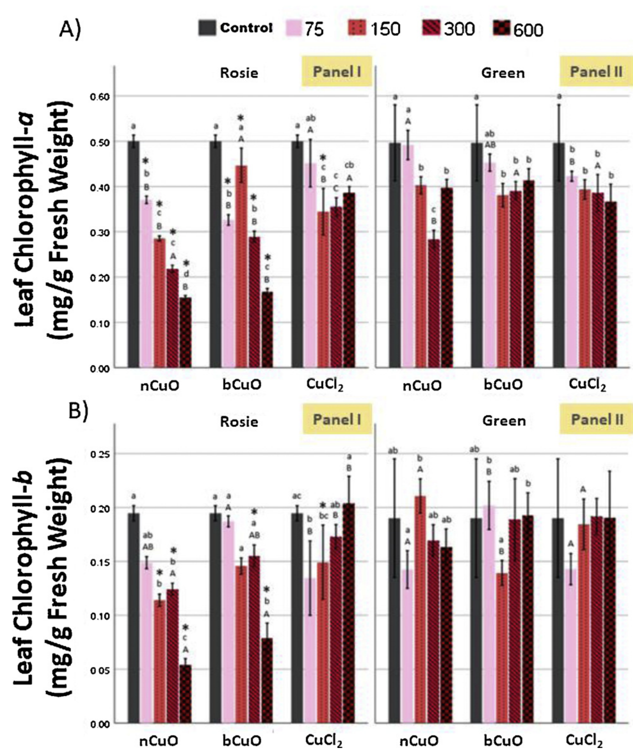

Fig. 3. Chlorophyll-*a* (A) and -*b* (B) contents in leaves of Rosie and Green plants (70-day-old) cultivated in soil amended with 0, 75, 150, 300, and 600 mg/kg of nCuO, bCuO, and $CuCl_2$. Data are means of three replicates ± SE. Lowercase letters represent significant differences in chlorophyll content between different plants treated with the same compounds at different concentrations. Capital letters stand for statistical differences between different compounds at the same concentration. Significant differences between the two varieties are marked with "*" ($p \leq 0.05$, n = 3).

chlorophyll-*a*, less inhibition on chlorophyll-*b* production may be due to less Cu accumulation in Green leaves than Rosie´s.

Although Cu is an essential element related to the electron transmission, plastocyanin activity as well as cytochrome-c oxidase, its excess could be harmful to the plants' photosynthesis process, since they may inhibit the synthesis of protochlorophyllide reductase and reduce the activity of photosynthesis-related enzymes (Sağlam et al., 2016). It has been reported that Cu^{2+} could perturb chloroplast functions and interfere with energy-transfer mechanisms (Liotenberg et al., 2015; Sağlam et al., 2016). In agreement with our findings, Zuverza-Mena et al. (2015) reported that chlorophyll content was decreased in plants treated with bCuO at 20 mg/kg. In another study, Shi et al. (2011) found similar results when they analyzed the chlorophyll content in duckweed (*Landoltia punctata*) treated with nCuO at 1 mg/L. Nair et al. (2014) also discovered that mung bean chlorophyll was reduced after exposing plants to nano CuO treatments at 100, 200, 500 mg/L for 21 days. Other studies also demonstrated that nCuO could affect chlorophyll in plants (Shaw et al., 2014; Trujillo-Reyes et al., 2014). In the present study, the decrease of chlorophyll contents may be involved in the growth reduction of bok choy plants.

3.4. Physiological impairments are distinctively associated with Cu-based compounds treatments

3.4.1. Leaves dry weight and foliar area

Dry leaves biomass of Rosie and Green treated plants is shown in Fig. 4A. Leaves are the central edible part of the bok choy plant; thus, it is among the essential index to measure. In Rosie cultivars, nCuO and $CuCl_2$ treatments at all concentrations and bCuO at ≥150 mg/kg significantly reduced leaves dry weight in comparison to control (Fig. 4A, Panel I) ($p \leq 0.05$). In Green variety, however, only the two highest concentrations of all Cu treatments showed significant responses (Fig. 4A, Panel II). The reduction in biomass could be related to the effect of Cu^{2+} and potential small particle size accumulation, as an excess of Cu impedes cell energy metabolism (Mustafa and Komatsu, 2016). Similar findings were reported by Du et al. (2018) as they demonstrated that nCuO and bCuO had harmful effects on oregano root growth and reduced shoot biomass by 21.6–58.5 %, compared to control. In other studies, the growth inhibition caused by nCuO exposure on alfalfa and lettuce (Hong et al., 2015), bean (Dimkpa et al., 2015), and cotton (Le Van et al., 2016) has been reported.

Rosie varieties basically exhibited more significant DW reduction percentages compared to their Green analogs at all Cu-based treatment exposures. This indicated that high-anthocyanin Rosie variety, which stored higher Cu quantities in leaves (Fig. 1A) and in roots (Fig. 1B), were more susceptible to Cu-based compounds. As discussed above, anthocyanins are capable to complex Cu compounds, which may explain the higher Cu contents found in Rosie plants. Previous literature has reported that the toxicity of nCuO or bCuO to plants was associated with hydrogen peroxide production, which led to oxidative stress (Da Costa and Sharma, 2016; Nair and Chung, 2015, 2014). Thus, more reactive oxygen species should have been produced in Rosie plants in response to Cu treatments.

The foliar area of both Rosie and Green varieties was reduced by Cu-based compounds compared with the corresponding controls (Fig. 4B). This effect was magnified with increasing exposure concentrations. As the DW parameter, the foliar area was more reduced after Cu treatments in Rosie varieties than Green cultivars.

3.4.2. Sugar and starch

Total sugar in leaves of bok choy plants treated with different Cu compounds is shown in Fig. 4C. Sugar content was not significantly altered by nCuO or $CuCl_2$ at any concentration ($p \leq 0.05$). Singularly, Rosie leaves treated with bCuO, at 150, 300, 600 mg/kg, had higher total sugar contents, with significant increases of 57 %, 52 %, and 49 %, respectively, compared with control (Fig. 4C, Panel I). Conversely, total sugar content in Green leaves was significantly decreased by bulk CuO at 600 mg/kg, compared to control (Fig. 4C, Panel II). The enhancement of total sugar content in bCuO-treated Rosie plants could be attributed to osmotic stress (Irigoyen et al., 1992; Oliveira et al., 2009; Paramithiotis and Patra, 2019). Sugar was possibly accumulated to overcome the external stress caused by Cu-based treatments. The different responses by Rosie and Green to bCuO may be related to their different anthocyanin content and Cu uptake.

3.5. Element accumulation

The concentration and reduction/increasing percentage of significantly affected macro (K and P) and microelements (Fe and Zn) in Rosie and Green plant tissues are shown in Table 1. No significant changes were found for B, Ca, Mg, Mn, Mo, and S contents, compared to control. Thus, they were not further examined.

3.5.1. Potassium

In root tissues from Green variety exposed to 600 mg/kg of nCuO, K concentration was significantly ~40 % lowered compared with control. The possible combination of the negative charge and higher surface to volume ratio displayed by nCuO might induce a complexation with K^{+} reducing their availability for plants uptake. The content of K in leaves from Rosie variety was significantly reduced by 45 % at 150 mg/kg of nCuO compared to control ($p \leq 0.05$). Contrarily, K in Green variety leaves was unaffected by any treatment. Leaves are the main reservoir for anthocyanin content (Jeon et al., 2018). Thus, their higher concentration in Rosie varieties might affect K accumulation. Additionally, Rosie leaves are more acidic compared to Green´s, which may contribute in regulating the activity of K uptake HKT1 transporters in bok choy plants (Schachtman and Schroeder, 1994).

3.5.2. Phosphorus

In both varieties, P was only altered in roots ($p \leq 0.05$). In Rosie

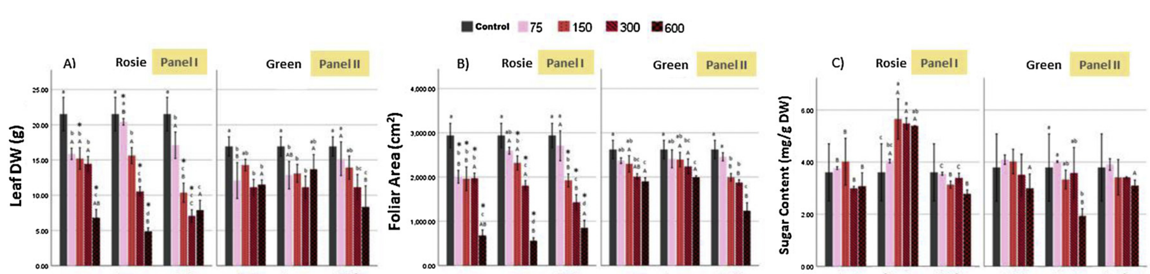

Fig. 4. Dry weight (A), foliar area (B), and sugar content (C) of leaves of 70-day-old Rosie and Green varieties cultivated in soil treated with 0, 75, 150, 300, and 600 mg/kg of nCuO, bCuO, and $CuCl_2$. Data are means of three replicates ± SE. Capital letters stand for statistical differences between different compounds at the same concentration. Lowercase letters represent significant differences between different concentrations of the same compounds. Significant differences between the two varieties are denoted with "*" ($p \leq 0.05$, n = 3).

plants, only nCuO at 600 mg/kg, significantly reduced P content by 38 % in comparison with control. Meanwhile, Green plants exhibited a 47 % P reduction at the same experimental conditions. A root P reduction of ~57 % in Green plants treated with both nCuO and bCuO at 150 mg/kg was also observed. This diminishment might occur due to either membrane transporters physical blockage caused by nCuO and bCuO particles, or by the formation of Cu phosphates compounds via released Cu^{2+} and phosphate ions in soil ($H_2PO_4^-$, HPO_4^{2-}) (Zuverza-Mena et al., 2015). Rawat et al. (2018) reported similar results in bell peppers as they found that bCuO at 500 mg/kg reduced P in roots by 36 % compared with the control. According to Apodaca et al. (2017) the P content of bean plants was reduced from 33 % to 97 % with bCuO and $CuCl_2$, respectively. Hong et al. (2015) found a reduction of root P in lettuce and alfalfa plants exposed to bCuO and nCuO at 5, 10, 20 mg/L. Pht1 root transporters are specific for P uptake, while Pht2 transporters are responsible for P translocation to the aerial part of plants (Buchner et al., 2004). It was possible that nCuO and bCuO down-regulated Pht1 molecules.

3.5.3. Iron

Iron content was significantly affected by Cu-based compounds in roots but not in leaves in the two varieties. In Rosie roots treated with bCuO at 75 mg/kg and $CuCl_2$ at 75 and 600 mg/kg, Fe accumulation was decreased by 30 %, 18 %, and 50 %, respectively, compared with control. Meanwhile, roots from Green plants significantly reduced their Fe content by 27 % when they were treated with ≥150 mg/kg of nCuO compared with control. According to previous studies, an uptake channel competition exists between Cu and Fe (Hong et al., 2015). Thus, Cu may displace Fe and inhibit its entrance (SCHMIDT et al., 1997). Besides, it is possible that the external Cu-based treatments inhibited proteins involved in Fe chelation (PUIG et al., 2007). This helps to explain how Fe content in root tissue was diminished. Our results were in agreement with previous findings. For instance, Apodaca et al. (2017) found that nCuO-treated bean seeds significantly lowered their Fe content by ~29 % compared with control. Ochoa et al. (2017) reported that leaves Fe of bean plants was decreased after nCuO exposure. Hong et al. (2015) reported that all particulate and ionic Cu-based compounds decreased Fe over 50 % in lettuce shoots, compared with control. Iron and anthocyanin contents were reported to be positively correlated in lilies (*Lilium*) (Parandian and Samavat, 2012). Nevertheless, in our study we did not find decreases in Fe content in leaves from Rosie and Green plants exposed to the Cu treatments, which suggest that anthocyanins might not be involved in Fe mobilization.

3.5.4. Zinc

Nearly all Cu-based compounds, at ≥300 mg/kg, decreased leaves Zn accumulation in both Rosie and Green plants. For instance, nCuO, at 300 mg/kg, significantly lowered leaves Zn by ~21 %, compared to control in Rosie variety. In Green plants exposed to $CuCl_2$, nCuO, and bCuO at 600 mg/kg, Zn content was significantly reduced by 25 %, 37 %, and 23 %, respectively, with respect to control.

Both Zn and Cu are known to share the same transporters of the ZIP family (Liu et al., 2019; Trujillo-Reyes et al., 2014). In this study, we observed that the Cu accumulation in leaves correlated with a falling in Zn content at the evaluated experimental conditions. A possible explanation would be that Cu ions are being preferably transported inside cells instead of Zn atoms. Similar results have previously been reported (Zuverza-Mena et al., 2015). Rawat et al. (2018) mentioned the reduction of Zn by 55 % in leaves and 47 % in fruits of bell pepper plants treated with Cu particles, compared to control.

4. Conclusion

This study was performed to evaluate the toxicological effects of three Cu-based compounds on two different bok choy varieties. The Cu content increase in tissues was dependent on the CuO particle size, Cu treatment concentration, and the bok choy variety. It was found a high level of Cu translocation from roots to edible aerial parts, which suggests an environmental risk, and further considerations for safe CuO NPs disposal should be considered. Rosie cultivars had more Cu than the green phenotypes and Cu-based compounds inhibited the growth of Rosie bok choy to a greater extent compared to Greens. Different factors such as specific activation of stress-responsive genes and the anthocyanin content, among others, may contribute to the different responses exhibited by Rosie and Green plants under Cu stress. The plant growth and nutrient element accumulation were reduced after Cu exposure. The nCuO caused more physiological impairments compared with bCuO and $CuCl_2$ treatments, suggesting nano-size-specific toxicology. Two-photon microscopy demonstrated the uptake, translocation, and most interestingly, the distribution of Cu inside bok choy leaf tissues. We found more Cu signal in the midrib than mesophyll from Rosie plants treated with nCuO; while more Cu intensity was found in the mesophyll than midrib of bCuO-treated Rosie plants. The varieties of bok choy plant significantly influenced the biodistribution of Cu, in addition to the applied CuO particle sizes. These results increase the understanding of nanoparticle uptake and translocation mechanisms in plant edible parts, and also provide valuable information for the application of nanomaterials in delivery systems for plant fertilizers or drugs in the future. Forthcoming experiments should consider Cation-Exchange Capacity measurements to evaluate the number of cations absorbed by the soil particles and also investigate the aggregation properties of nCuO in water or plant extracts. Further work is required to ensure a sustainable application of nCuO in agriculture and safe disposal into the environment.

Author contributions

Chaoyi Deng, Yi Wang, Jose R. Peralta-Videa, Jose A. Hernandez-Viezcas, and Jorge Gardea-Torresdey conceptualized the investigation and planned the experiments. Chaoyi Deng, Yi Wang, Andres Reyes, Chunqiang Li, and Reagan S. Turley conducted the research and investigation process. Keni Cota-Ruiz, Youping Sun, Jose R. Peralta-Videa, Jose A. Hernandez-Viezcas, Genhua Niu, and Jorge Gardea-Torresdey contributed to the visualization and supervision. Chaoyi Deng, Yi Wang, prepared the original draft. Keni Cota-Ruiz, and Jorge Gardea-Torresdey reviewed and edited the manuscript. Jorge Gardea-Torresdey acquired the financial support.

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

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References

Abdallah, B.B., Zhang, X., Andreu, I., Gates, B.D., El Mokni, R., Rubino, S., Landoulsi, A., Chatti, A., 2020. Differentiation of nanoparticles isolated from distinct plant species naturally growing in a heavy metal polluted site. J. Hazard. Mater. 386, 121644. https://doi.org/10.1016/j.jhazmat.2019.121644.

Abdel-Wahab, D.A., Othman, N.A.R.M., Hamada, A.M., 2019. Effects of copper oxide nanoparticles to *Solanum nigrum* and its potential for phytoremediation. Plant Cell Tissue Organ Cult. 1–15. https://doi.org/10.1007/s11240-019-01588-5.

Acosta, Y., Zhang, Q., Rahaman, A., Ouellet, H., Xiao, C., Sun, J., Li, C., 2014. Imaging cytosolic translocation of Mycobacteria with two-photon fluorescence resonance energy transfer microscopy. Biomed. Opt. Express 5, 3990. https://doi.org/10.1364/BOE.5.003990.

Andrew, Mc Williams, 2018. Nanocomposites, nanoparticles, nanoclays | BCC research [WWW document]. BCC Res. https://www.bccresearch.com/market-research/nanotechnology/nanocomposites-nanoparticles-nanoclays-and-nanotubes-global-markets.html.

Anjum, N.A., Adam, V., Kizek, R., Duarte, A.C., Pereira, E., Iqbal, M., Lukatkin, A.S., Ahmad, I., 2015. Nanoscale copper in the soil–plant system – toxicity and underlying potential mechanisms. Environ. Res. 138, 306–325. https://doi.org/10.1016/J.ENVRES.2015.02.019.

Apodaca, S.A., Tan, W., Dominguez, O.E., Hernandez-Viezcas, J.A., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2017. Physiological and biochemical effects of nanoparticulate copper, bulk copper, copper chloride, and kinetin in kidney bean (*Phaseolus vulgaris*) plants. Sci. Total Environ. 599–600, 2085–2094. https://doi.org/10.1016/j.scitotenv.2017.05.095.

Bonilla-Bird, N.J., Paez, A., Reyes, A., Hernandez-Viezcas, J.A., Li, C., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2018. Two-photon microscopy and spectroscopy studies to determine the mechanism of copper oxide nanoparticle uptake by sweetpotato roots during postharvest treatment. Environ. Sci. Technol. 52, 9954–9963. https://doi.org/10.1021/acs.est.8b02794.

Boulton, R., 2001. The copigmentation of anthocyanins and its role in the color of red wine: a critical review. Am. J. Enol. Vitic. 52 67 LP – 87.

Buchner, P., Takahashi, H., Hawkesford, M.J., 2004. Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport. J. Exp. Bot. 55, 1765–1773. https://doi.org/10.1093/jxb/erh206.

Cortez, R., Luna-Vital, D.A., Margulis, D., Gonzalez de Mejia, E., 2017. Natural pigments: stabilization methods of anthocyanins for food applications. Compr. Rev. Food Sci. Food Saf. 16, 180–198. https://doi.org/10.1111/1541-4337.12244.

Da Costa, M.V.J., Sharma, P.K., 2016. Effect of copper oxide nanoparticles on growth, morphology, photosynthesis, and antioxidant response in *Oryza sativa*. Photosynthetica 54, 110–119. https://doi.org/10.1007/s11099-015-0167-5.

Dai, L.P., Dong, X.J., Ma, H.H., 2012. Antioxidative and chelating properties of anthocyanins in *Azolla imbricata* induced by cadmium. Polish J. Environ. Stud. 21, 837–844.

DalCorso, G., Manara, A., Piasentin, S., Furini, A., 2014. Nutrient metal elements in plants. Metallomics 6, 1770–1788. https://doi.org/10.1039/C4MT00173G.

Dimkpa, C.O., Latta, D.E., McLean, J.E., Britt, D.W., Boyanov, M.I., Anderson, A.J., 2013. Fate of CuO and ZnO nano- and microparticles in the plant environment. Environ. Sci. Technol. 47, 4734–4742. https://doi.org/10.1021/es304736y.

Dimkpa, C.O., McLean, J.E., Britt, D.W., Anderson, A.J., 2015. Nano-CuO and interaction with nano-ZnO or soil bacterium provide evidence for the interference of nanoparticles in metal nutrition of plants. Ecotoxicology 24, 119–129. https://doi.org/10.1007/s10646-014-1364-x.

Du, W., Tan, W., Yin, Y., Ji, R., Peralta-Videa, J.R., Guo, H., Gardea-Torresdey, J.L., 2018. Differential effects of copper nanoparticles/microparticles in agronomic and physiological parameters of oregano (*Origanum vulgare*). Sci. Total Environ. 618, 306–312. https://doi.org/10.1016/j.scitotenv.2017.11.042.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356. https://doi.org/10.1021/ac60111a017.

Elemike, E.E., Uzoh, I.M., Onwudiwe, D.C., Babalola, O.O., 2019. The role of nanotechnology in the fortification of plant nutrients and improvement of crop production. Appl. Sci. 9, 1–32. https://doi.org/10.3390/app9030499.

European Commission, 2018. Science for Environment Police.

Gao, X., Avellan, A., Laughton, S., Vaidya, R., Rodrigues, S.M., Casman, E.A., Lowry, G.V., 2018. CuO nanoparticle dissolution and toxicity to wheat (*Triticum aestivum*) in Rhizosphere soil. Environ. Sci. Technol. 52, 2888–2897. https://doi.org/10.1021/acs.est.7b05816.

He, Q., Zhang, Z., Zhang, L., 2016. *Brassica rapa*anthocyanin accumulation, antioxidant ability and stability, and a transcriptional analysis of anthocyanin biosynthesis in purple heading Chinese cabbage (L. ssp. pekinensis). J. Agric. Food Chem. 64, 132–145. https://doi.org/10.1021/acs.jafc.5b04674.

Helmchen, F., Denk, W., 2005. Deep tissue two-photon microscopy. Nat. Methods 2. https://doi.org/10.1038/NMETH818.

Hong, J., Rico, C.M., Zhao, L., Adeleye, A.S., Keller, A.A., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2015. Toxic effects of copper-based nanoparticles or compounds to lettuce (Lactuca sativa) and alfalfa (*Medicago sativa*). Environ. Sci. Process. Impacts 17, 177–185. https://doi.org/10.1039/C4EM00551A.

Hosseinian, F.S., Beta, T., 2007. Saskatoon and wild blueberries have higher anthocyanin contents than other Manitoba berries. J. Agric. Food Chem. 55, 10832–10838. https://doi.org/10.1021/jf072529m.

Irigoyen, J.J., Einerich, D.W., Sánchez-Díaz, M., 1992. Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiol. Plant. 84, 55–60. https://doi.org/10.1111/J.1399-3054.1992.TB08764.X.

Jeon, J., Lim, C.J., Kim, J.K., Park, S.U., 2018. Comparative metabolic profiling of green and purple pakchoi *(Brassica rapa* subsp. chinensis). Molecules 23. https://doi.org/10.3390/molecules23071613.

Keller, A.A., McFerran, S., Lazareva, A., Suh, S., 2013. Global life cycle releases of engineered nanomaterials. J. Nanopart. Res. 15, 1692. https://doi.org/10.1007/s11051-013-1692-4.

Kulikowska, D., Gusiatin, Z.M., Bułkowska, K., Klik, B., 2015. Feasibility of using humic substances from compost to remove heavy metals (Cd, Cu, Ni, Pb, Zn) from contaminated soil aged for different periods of time. J. Hazard. Mater. 300, 882–891. https://doi.org/10.1016/J.JHAZMAT.2015.08.022.

Le Van, N., Ma, C., Shang, J., Rui, Y., Liu, S., Xing, B., 2016. Effects of CuO nanoparticles on insecticidal activity and phytotoxicity in conventional and transgenic cotton. Chemosphere 144, 661–670. https://doi.org/10.1016/j.chemosphere.2015.09.028.

Liotenberg, S., Steunou, A.-S., Durand, A., Bourbon, M.-L., Bollivar, D., Hansson, M., Astier, C., Ouchane, S., 2015. Oxygen-dependent copper toxicity: targets in the chlorophyll biosynthesis pathway identified in the copper efflux ATPase CopA deficient mutant. Environ. Microbiol. 17, 1963–1976. https://doi.org/10.1111/1462-2920.12733.

Liu, X.S., Feng, S.J., Zhang, B.Q., Wang, M.Q., Cao, H.W., Rono, J.K., Chen, X., Yang, Z.M., 2019. OsZIP1 functions as a metal efflux transporter limiting excess zinc, copper and cadmium accumulation in rice. BMC Plant Biol. 19, 283. https://doi.org/10.1186/s12870-019-1899-3.

Lv, J., Christie, P., Zhang, S., 2018. Uptake, translocation, and transformation of metal-based nanoparticles in plants: recent advances and methodological challenges. Environ. Sci. Nano 22–25. https://doi.org/10.1039/C8EN00645H.

Mustafa, G., Komatsu, S., 2016. Toxicity of heavy metals and metal-containing nanoparticles on plants. Biochim. Biophys. Acta 1864, 932–944. https://doi.org/10.1016/j.bbapap.2016.02.020.

Nair, P.M.G., Chung, I.M., 2014. Impact of copper oxide nanoparticles exposure on *Arabidopsis thaliana* growth, root system development, root lignificaion, and molecular level changes. Environ. Sci. Pollut. Res. 21, 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.

Nalci, O.B., Nadaroglu, H., Pour, A.H., Gungor, A.A., Haliloglu, K., 2019. Effects of ZnO, CuO and γ-Fe3O4 nanoparticles on mature embryo culture of wheat (*Triticum aestivum* L.). Plant Cell Tissue Organ Cult. 136, 269–277. https://doi.org/10.1007/s11240-018-1512-8.

Ochoa, L., Medina-Velo, I.A., Barrios, A.C., Bonilla-Bird, N.J., Hernandez-Viezcas, J.A., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2017. Modulation of CuO nanoparticles toxicity to green pea (*Pisum sativum* Fabaceae) by the phytohormone indole-3-acetic acid. Sci. Total Environ. 598, 513–524. https://doi.org/10.1016/j.scitotenv.2017.04.063.

Oliveira, H., Barros, A.S., Delgadillo, I., Coimbra, M.A., Santos, C., 2009. Effects of fungus inoculation and salt stress on physiology and biochemistry of in vitro grapevines: emphasis on sugar composition changes by FT-IR analyses. Environ. Exp. Bot. 65, 1–10. https://doi.org/10.1016/J.ENVEXPBOT.2008.05.003.

Paramithiotis, S., Patra, J., 2019. Food Molecular Microbiology. CRC Press.

Parandian, F., Samavat, S., 2012. Effects of Fulvic and Humic acid on Anthocyanin, soluble Sugar, Amylase Enzyme and some micronurient elements in Lilium. Int. Res. J. Appl. Basic Sci. 3 (5), 924–929.

Pestovsky, Y.S., Martínez-Antonio, A., 2017. The use of nanoparticles and nanoformulations in agriculture. J. Nanosci. Nanotechnol. 17, 8699–8730. https://doi.org/10.1166/jnn.2017.15041.

Porra, R.J., 2002. The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth. Res. 73, 149–156. https://doi.org/10.1023/A:1020470224740.

Primikyri, A., Mazzone, G., Lekka, C., Tzakos, A.G., Russo, N., Gerothanassis, I.P., 2015. Understanding zinc(II) chelation with quercetin and luteolin: a combined NMR and theoretical study. J. Phys. Chem. B 119, 83–95. https://doi.org/10.1021/jp509752s.

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, 271–290. https://doi.org/10.1111/j.1365-3040.2007.01642.x.

Rajput, V., Minkina, T., Fedorenko, A., Sushkova, S., Mandzhieva, S., Lysenko, V., Duplii, N., Fedorenko, G., Dvadnenko, K., Ghazaryan, K., 2018. Toxicity of copper oxide nanoparticles on spring barley (*Hordeum sativum* distichum). Sci. Total Environ. 645, 1103–1113. https://doi.org/10.1016/j.scitotenv.2018.07.211.

Rawat, S., Pullagurala, V.L.R., Hernandez-Molina, M., Sun, Y., Niu, G., Hernandez-Viezcas, J.A., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2018. Impacts of copper oxide nanoparticles on bell pepper *(Capsicum annum* L.) plants: a full life cycle study. Environ. Sci. Nano 5, 83–95. https://doi.org/10.1039/C7EN00697G.

Rochfort, Simone J., Imsic, Michael, Rod Jones, ‡, Craige Trenerry, V., Tomkins, B., 2006. Characterization of flavonol conjugates in immature leaves of Pak Choi [*Brassica rapa* L. Ssp. chinensis L. (Hanelt.)] by HPLC-DAD and LC-MS/MS. J. Agric. Food Chem. 53, 4855–4860. https://doi.org/10.1021/JF060154J.

Sağlam, A., Yetişsin, F., Demiralay, M., Terzi, R., 2016. Copper Stress and Responses in Plants, Plant Metal Interaction. Elsevier, pp. 21–40. https://doi.org/10.1016/B978-0-12-803158-2.00002-3.

Schachtman, D.P., Schroeder, J.I., 1994. Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. Nature 370, 655–658. https://doi.org/10.1038/370655a0.

Schmidt, W., Bartels, M., Tittel, J., Fuhner, C., 1997. Physiological effects of copper on iron acquisition processes in Plantago. New Phytol. 135, 659–666. https://doi.org/10.1046/j.1469-8137.1997.00691.x.

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

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, 1277–1282. https://doi.org/10.1016/j.envpol.2011.01.028.

Spielman-Sun, E., Lombi, E., Donner, E., Avellan, A., Etschmann, B., Howard, D., Lowry, G.V., 2018. Temporal evolution of copper distribution and speciation in roots of *Triticum aestivum* exposed to CuO, $Cu(OH)_2$, and CuS nanoparticles. Environ. Sci. Technol. 52, 9777–9784. https://doi.org/10.1021/acs.est.8b02111.

Tan, W., Gao, Q., Deng, C., Wang, Y., Lee, W.-Y., Hernandez-Viezcas, J.A., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2018. Foliar exposure of $Cu(OH)_2$ nanopesticide to basil (*Ocimum basilicum*): variety dependent copper translocation and biochemical responses. J. Agric. Food Chem. 66, 3358–3366. https://doi.org/10.1021/acs.jafc.8b00339.

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, 255–263. https://doi.org/10.1016/j.jhazmat.2013.11.067.

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

Wang, T., Li, C., Liu, Y., Li, T., Zhang, J., Sun, Y., 2015. Inhibition effects of Chinese cabbage powder on aflatoxin B1-induced liver cancer. Food Chem. 186, 13–19. https://doi.org/10.1016/J.FOODCHEM.2015.02.138.

Welch, C.R., Wu, Q., Simon, J.E., 2008. Recent advances in anthocyanin analysis and characterization. Curr. Anal. Chem. 4, 75–101.

Yang, X., Pan, H., Wang, P., Zhao, F.J., 2017a. Particle-specific toxicity and bioavailability of cerium oxide (CeO_2) nanoparticles to *Arabidopsis thaliana*. J. Hazard. Mater. 322, 292–300. https://doi.org/10.1016/j.jhazmat.2016.03.054.

Yang, Y., Christakos, G., Guo, M., Xiao, L., Huang, W., 2017b. Space-time quantitative source apportionment of soil heavy metal concentration increments. Environ. Pollut. 223, 560–566. https://doi.org/10.1016/J.ENVPOL.2017.01.058.

Zhang, Y., Chen, G., Dong, T., Pan, Y., Zhao, Z., Tian, S., Hu, Z., 2014. Anthocyanin Accumulation and Transcriptional Regulation of Anthocyanin Biosynthesis in Purple Bok Choy (*Brassica rapa*var. chinensis). J. Agric. Food Chem. 62, 12366–12376. https://doi.org/10.1021/jf503453e.

Zuverza-Mena, N., Medina-Velo, I.A., Barrios, A.C., Tan, W., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2015. Copper nanoparticles/compounds impact agronomic and physiological parameters in cilantro (*Coriandrum sativum*). Environ. Sci. Process. Impacts 17, 1783–1793. https://doi.org/10.1039/C5EM00329F.