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Citation

Xiao, Y., Ma, J., Xian, J., Peijnenburg, W. J. G. M., Du, Y., Tian, D., ... Lihua, T. (2022). Copper accumulation and physiological markers of soybean (Glycine max) grown in agricultural soil amended with copper nanoparticles. *Ecotoxicology And Environmental Safety*, *229*. doi:10.1016/j.ecoenv.2021.113088

Ecotoxicology and Environmental Safety 229 (2022) 113088

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Copper accumulation and physiological markers of soybean (*Glycine max*) grown in agricultural soil amended with copper nanoparticles

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ARTICLE INFO

Edited by Dr R Pereira

Keywords:
Copper nanoparticles
$CuSO_4$
Accumulation
Physiological effects
Soybean

ABSTRACT

Copper-based nanoparticles (NPs) display a strong potential to replace copper salts (e.g., $CuSO_4$) for application in agricultures as antimicrobial agents or nutritional amendments. Yet, their effects on crop quality are still not comprehensively understood. In this study, the Cu contents in soybeans grown in soils amended with Cu NPs and $CuSO_4$ at 100–500 mg Cu/kg and the subsequent effects on the plant physiological markers were determined. The Cu NPs induced 29–89% at the flowering stage (on Day 40) and 100–165% at maturation stage (on Day 100) more Cu accumulation in soybeans than $CuSO_4$. The presence of particle aggregates in the root cells with deformation upon the Cu NP exposure was observed by transmission electron microscopy. The Cu NPs at 100 and 200 mg/kg significantly improved the plant height and biomass, yet significantly inhibited at 500 mg/kg, compared to the control. In leaves chlorophyll-*b* was more sensitive than chlorophyll-*a* and carotenoids to the Cu NP effect. The Cu NPs significantly decreased the root nitrogen and phosphorus contents, while they significantly increased the leaf potassium content in comparison with control. Our results imply that cautious use of Cu NPs in agriculture is warranted due to relatively high uptake of Cu and altered nutrient quality in soybeans.

1. Introduction

Engineered nanoparticles (NPs) have emerged as an extremely important group of materials due to their unique physicochemical properties. Metal-based nanoparticles (MNPs) are among the most highly produced and used NPs, accounting for 37% of the total number of nanoproducts on the global market (Vance et al., 2015). With the widespread production and application of MNPs, the consequence that they are released to the environment is inevitable. Concern regarding their effects on environments and human health has therefore grown in recent years. As one of the most commonly manufactured MNPs, Cu-based NPs have been increasingly used in a variety of products such as pesticides, fungicides, catalysts, batteries, plastics, and antifouling agents (Anjum et al., 2015a; Mary et al., 2009). Soil is an important sink for Cu-based NPs. It is estimated that around 200 tons of Cu-based NPs are being manufactured globally every year and at least 36 tons of them are annually released to soils (Keller et al., 2013; Keller and Lazareva, 2014). Furthermore, NPs tend to experience far longer residence times in soils than in other environmental compartments (Peijnenburg et al., 2016). Hence, there is a necessity to thoroughly assess the influence of Cu-based NPs on soil ecosystems in order to comprehensively understand their environmental impacts.

Plants are a vital component of the soil ecosystem and they may serve as potential carriers of MNPs following their bioaccumulation and biomagnification in the food chain (Bayat et al., 2014; Gardea-Torresdey et al., 2014). It is therefore important to monitor the accumulation of MNPs in plants. Cu is an essential micronutrient for plant growth and development (Nekrasova et al., 2011; Yruela, 2005). Both deficiency and an excess of Cu can be detrimental for plants (Anjum et al., 2015b). It is well known that metal ions can be taken up through the transport channels in or on plant cell membranes (Yang et al., 2018; Korshunova et al., 1999). However, knowledge on the accumulation of metal

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https://doi.org/10.1016/j.ecoenv.2021.113088
Received 25 May 2021; Received in revised form 5 December 2021; Accepted 12 December 2021

compounds in the form of NPs in plants is still limited. According to the existing studies involved in determination of the accumulation of MNPs in plants, it seems plausible that NPs even with a primary size larger than the exclusion limit of cell wall pores could be accumulated and transported in plants (Rossi et al., 2018; Schwabe et al., 2013; Laure et al., 2012; Harris and Bali, 2008). However, most of the related studies have been conducted in homogeneous media, such as water, sand and agar. Soils are highly heterogeneous and commonly solid-dominated matrices are composed of organic and inorganic aggregates with different size (Tisdall and Oades, 1979). The accumulation capacity of MNPs in plants grown in soil may vastly differ from accumulation in the same plant yet cultivated in homogeneous compartments. The current data on the accumulation of MNPs in terrestrial plants grown in soil compartments are limited, let alone the data on comparing the accumulation of ionic and nanoparticulate metal compounds in plants grown in soil. These knowledge gaps obstruct the safety assessment and sustainable use of MNPs.

A range of studies have been devoted to determining the physiological effects of MNPs on edible plants, with both negative and positive effects on physiological parameters being reported (Wan et al., 2019; Rui et al., 2018; Zhang et al., 2018; Singh et al., 2017; Shaw et al., 2014). It has been well demonstrated that the specific effects of MNPs on plant physiology is dependent on species of plants (Deng et al., 2020; Achari and Kowshik, 2018). As the fifth largest production of crop, soybean (*Glycine max*) contributes about 40% of the annual global oilseed crop (Priester et al., 2012). Some existing data have suggested that soybeans are vulnerable to MNPs. For example, Lopez-Moreno et al. (2010) found that even though CeO_2 NPs did not affect soybean germination, they could enter into root cells and cause DNA damage. Priester et al. (2012) found that ZnO NPs significantly increased the Zn concentration in soybean leaves and beans, and CeO_2 NPs impaired soybean growth by eliminating N_2 fixation potentials. Nonetheless, the effects of MNPs on soybean plants are not well-studied. For instance, information on the uptake of MNPs (particularly Cu-based NPs) by soybean plants, on the photosynthetic traits, and on nutrient quality of soybeans upon MNPs treatment is still lacking.

Therefore, the accumulation of nanoparticulate and ionic Cu and the subsequent physiological changes in soybeans were determined in this study. The primary objectives of this study were to: (1) differentiate the accumulation capacity of the nanoparticulate and ionic Cu in soybeans; (2) determine the effects of Cu NPs on the soybean physiology including growth parameters and contents of photosynthetic pigments and macronutrient elements. In addition, it is widely known that adsorption and accumulation are important with regard to the induction of effects on living organisms, while little effort has been made so far to correlate the metal content taken up by plants after exposure to MNPs with the physiological levels of the plants. This hinders proper elucidation of the mechanisms underlying the physiological effects of MNPs on plants. Hence, examining the correlation of Cu content in soybeans upon exposure to Cu NPs with subsequent physiological changes of the soybean was another objective of this study.

2. Materials and methods

2.1. Chemicals and characterization of Cu NPs

Cu NPs (purity: 99.9%; nominal size and shape: 10–30 nm and sphere, respectively) were purchased from Macklin Biochemical Co., Ltd (Shanghai, China). The shape and primary size in deionized water were characterized by transmission electron microscopy (TEM, JEM-1010, JEOL Inc.). Nano Measure 1.2 software (Fudan University, China) was applied to measure the size of the Cu NPs. Brunauer-Emmett-Teller (BET) method using N_2 adsorption apparatus (Micromeritics ASPA 2010) was used to determine the specific area of the Cu NPs. TEM image (Fig. S1) verified that the shape of the Cu NPs was spherical with a primary size of approximately 17 nm. The specific surface area obtained from the BET analysis for the Cu NPs was 29.69 m^2/g. $CuSO_4{\cdot}5H_2O$, HNO_3, H_2SO_4, H_2O_2 and other chemicals used in this study were at least analytically grade and were provided by Sinopharm Chemical Reagent Co., China.

2.2. Soil and treatments

The soil at a depth of 0–20 cm used in this study was collected from an agricultural field (latitude: 29°60’ N and longitude: 104°00’ E) in Renshou County, Sichuan Province, China. After air drying and removing stones and roots, the soil samples were sieved through a 2-mm nylon mesh. The background Cu concentration in the soil was 35.87 mg/kg. The physical and chemical properties of the experimental soil are presented in Table S1.

The soybeans were grown in the polyvinyl chloride (PVC) pots (with an inner diameter of 20 cm and a depth of 15 cm). Each pot contained 2.5 kg of the air-dried soil. The two Cu compounds (Cu NPs and $CuSO_4.5H_2O$) were added as powders into the air-dried soils to obtain the target concentrations of 100, 200, and 500 mg Cu per kg of soil. The concentration range of 100–500 mg Cu/kg was selected, as the Cu concentrations in natural soils are commonly in this range (Rawat et al., 2018; Yang et al., 2017b, 2017a; Kulikowska et al., 2015). The soil and Cu compounds were manually mixed for 30 min to ensure the homogeneity. Soil samples without addition of any Cu compounds were set as the control. Each treatment consisted of 4 replicates. Two important soybean growth stages (flowering and maturation stages) were taken to compare the effects and the Cu accumulation in the soybeans upon the Cu compound treatments. Therefore, we used eight pots for each treatment and half of them (as replicates) were grouped and harvested at the flowering and maturation stages, respectively. In total, 56 pots were used in this work (6 Cu compound treatments and a control × 4 replicates × 2 growth stages). Sufficient deionized water was added to the pots to impose the soil moisture at about 70% of field capacity. All the soil samples amended with different concentrations of Cu compound were equilibrated for 2 weeks before sowing the soybean seeds (Yan et al., 2013). The pH values of the soil samples upon different Cu treatments after the equilibration were monitored (Fig. S2).

The soybean seeds (*Glycine max* L. cv. Nandou 12) were obtained from Sichuan Engineering Research Center for Crop Strip Intercropping System (China). On 11 April, 2020, four seeds with similar size were sown at a depth of 3–5 cm in each pot and only two healthy-looking soybean plants with similar growth status were left in each pot on the seventh day after germination. All the pots were placed in a greenhouse. In the greenhouse, the temperature was approximately 30 and 20 ℃ during the day (16 h) and at night (8 h), respectively; the daily light integral was averagely 10 mol m^{-2} d^{-1}; the relative humidity was 50–75%. During the cultivation process, all the pots were irrigated with deionized water every other day to maintain the soil moisture at near 70% of field capacity.

2.3. Analysis of plant physiology

On Day 40 after sowing (at the flowering stage), the soybean plants were used to analyze the physiological parameters. The soybean plants were carefully taken out from the soils, washed with running tap water, rinsed with 0.01% HNO_3 and deionized water successively, and then dried with paper towels. The plant height and fresh biomass were recorded for each plant. The fresh plants were oven-dried at 70 ℃ for 3 d before weighing the dry biomass. Subsequently, the dried plant samples were divided into roots, stems and leaves, followed by digestion with H_2SO_4-H_2O_2. The Kjeldahl method was used to determine the nitrogen (N) content in the roots, leaves and stems (Grimshaw et al., 1989). The contents of phosphorus (P) and potassium (K) in different parts of the soybeans were analyzed by molybdenum blue colorimetry and a flame photometer, respectively (Thomas et al., 1967). The contents of photosynthetic pigments in the fresh leaves at the flowering stage (on Day 40) were analyzed. Briefly, 0.5 g of subapical leaves sampled from the soybean plants cultivated upon different Cu treatments were ground and then extracted with 80% acetone. The absorbance of pigment extract was determined at wavelengths of 663, 645 and 470 nm with a UV-Vis spectrophotometer (Perkin Elmer Lamda, Uberlinger, Germany). The contents of chlorophyll *a* (Chl-*a*) and *b* (Chl-*b*) as well as carotenoids were calculated according to the method of Lichtenthaler (1987).

2.4. Analysis of Cu content in soybeans

On Day 40 after sowing, the dried roots, stems and leaves were digested by plasma-pure HNO_3 (65%) and H_2O_2 (30%) (1: 4, v/v) until the digestion solution became transparent. The digests were added to 15 mL with deionized water and then the Cu concentrations in the different digested samples were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 4300 DV; Perkin-Elmer). Standard reference material 1570a acquired from the National Institute of Standard and Technology was used to validate the digestion and analytic method for Cu. The recovery rate of the Cu in the reference material was in the range of 96–102%. For quality control and assurance, a Cu standard was analyzed every 25 samples. TEM with energy dispersive X-ray spectroscopy (EDS) was used to confirm the presence of the Cu NPs inside the treated root cells and to observe the morphology of the soybean root cells upon the Cu NP treatments. The specific methods for TEM with EDS observation of the root cells were detailed in other studies (Hao et al., 2016; Du et al., 2015; Larue et al., 2012).

In order to monitor the Cu content in different organs (i.e., roots, stems and beans) at the maturation stage of the soybeans, the other set of soil samples amended with the Cu NPs and $CuSO_4$ at the concentration range from 0 to 500 mg/kg were used to grow the soybean plants from 11 April to 20 July, 2020. On Day 100 after sowing (at the maturation stage), the soybean plants were harvested, and then the Cu contents in the dried roots, stems and beans were measured by ICP-OES, as depicted above. The Cu content on Day 100 in the leaves was not determined, as at this stage most of the leaves had fallen down, and consequently there was no enough leaf sample for Cu analysis.

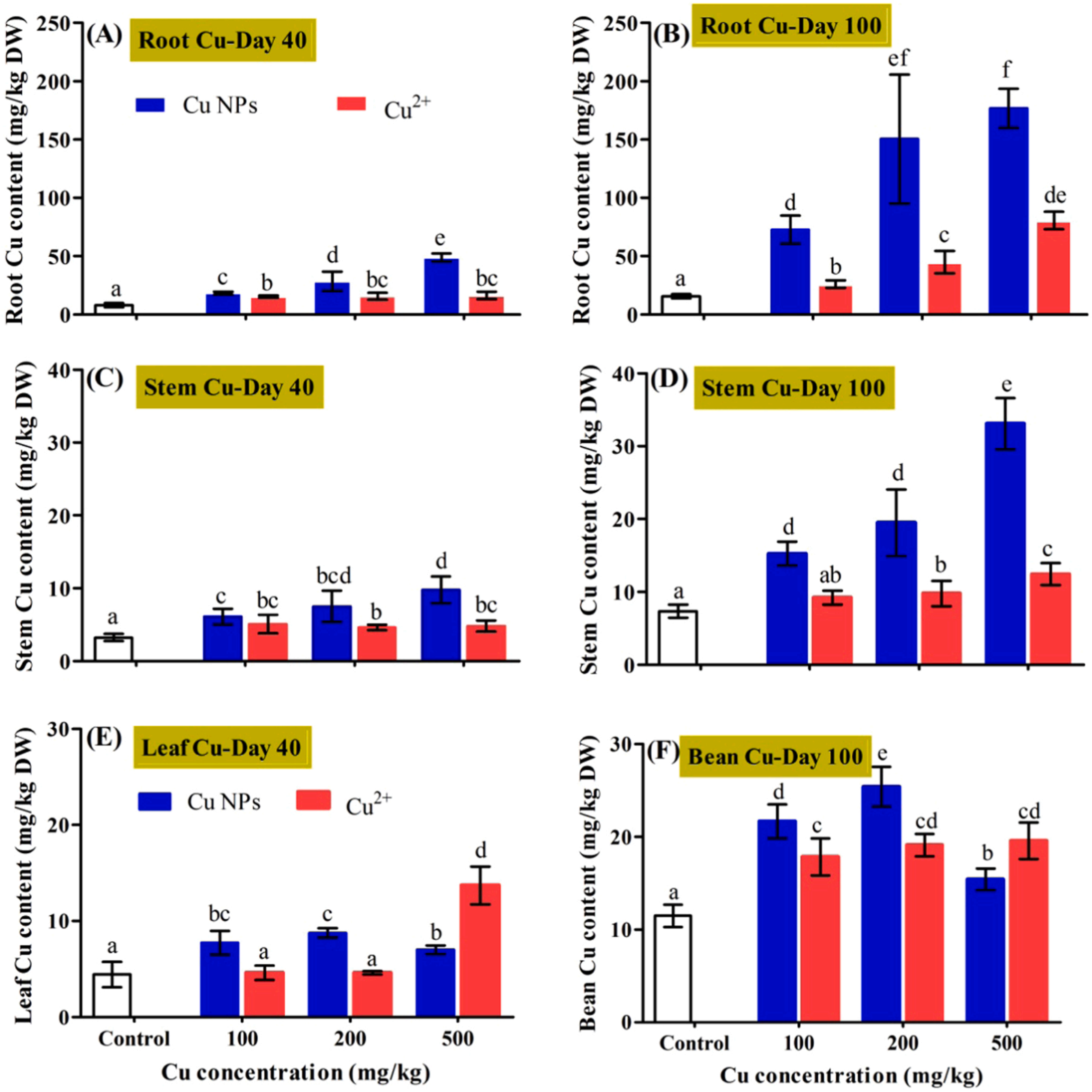

Fig. 1. Contents of Cu in different parts-roots (A and B), stems (C and D), leaves (E) and beans (F) of the soybeans at the flowering (left column-day 40th) and maturation (right column-day 100th) stages at 0, 100, 200 and 500 mg/kg of soil treatments with Cu NPs and Cu^{2+}. Data are presented as mean ± SD ($n = 4$). Different letters indicate significant differences among different treatments as tested by one-way ANOVA and *t*-test ($P < 0.05$).

2.5. *Statistical analysis*

Statistical analysis was performed using SPSS 19.0 for Windows (SPSS, 19.0 SPSS Inc., Chicago, IL, USA). The plant physiological parameters (including the plant height, biomass, the contents of photosynthetic pigments, macronutrients and Cu) upon the same Cu compound treatment with varying concentrations were compared using a one-way analysis variance (one-way ANOVA) with a Tukey's post hoc test. Before ANOVA was applied, the normality and homogeneity of variance of the data were checked. The differences of the physiological parameters between the Cu NP and $CuSO_4$ treatments were compared using independent sample *t*-test. The significance level in all analysis was set at $\alpha = 0.05$. Results in this study were expressed as mean ± standard deviation (SD). All test statistics (degrees of freedom, *P*-values and F-values) are listed in Table S2.

3. Results and discussion

3.1. *Cu contents taken up by soybeans*

Generally, both the Cu NPs and $CuSO_4$ significantly increased the total Cu contents in the soybeans, as compared to the control ($P < 0.05$) (Fig. 1). It was furthermore found that the Cu NPs induced 29–89% at the flowering stage (determined on Day 40 after cultivation) and 100–165% at the maturation stage (determined on Day 100 after cultivation) more Cu accumulation in the soybeans, as compared to the Cu accumulation upon the $CuSO_4$ treatments.

In roots, the Cu contents upon both the Cu NP and $CuSO_4$ treatments were significantly increased in comparison with the control ($P < 0.05$) (Fig. 1A and B). Furthermore, the Cu content in the soybean roots upon the Cu NP treatment was significantly higher than that upon the $CuSO_4$ treatment with an equivalent Cu level at both the flowering and maturation stages (on Days 40 and 100, respectively) (Fig. 1A and B). In order to demonstrate whether the particulate Cu could be internalized into the soybean root cells, TEM with EDS analysis was performed. The root cells of the control were intact and well-shaped and no particles were

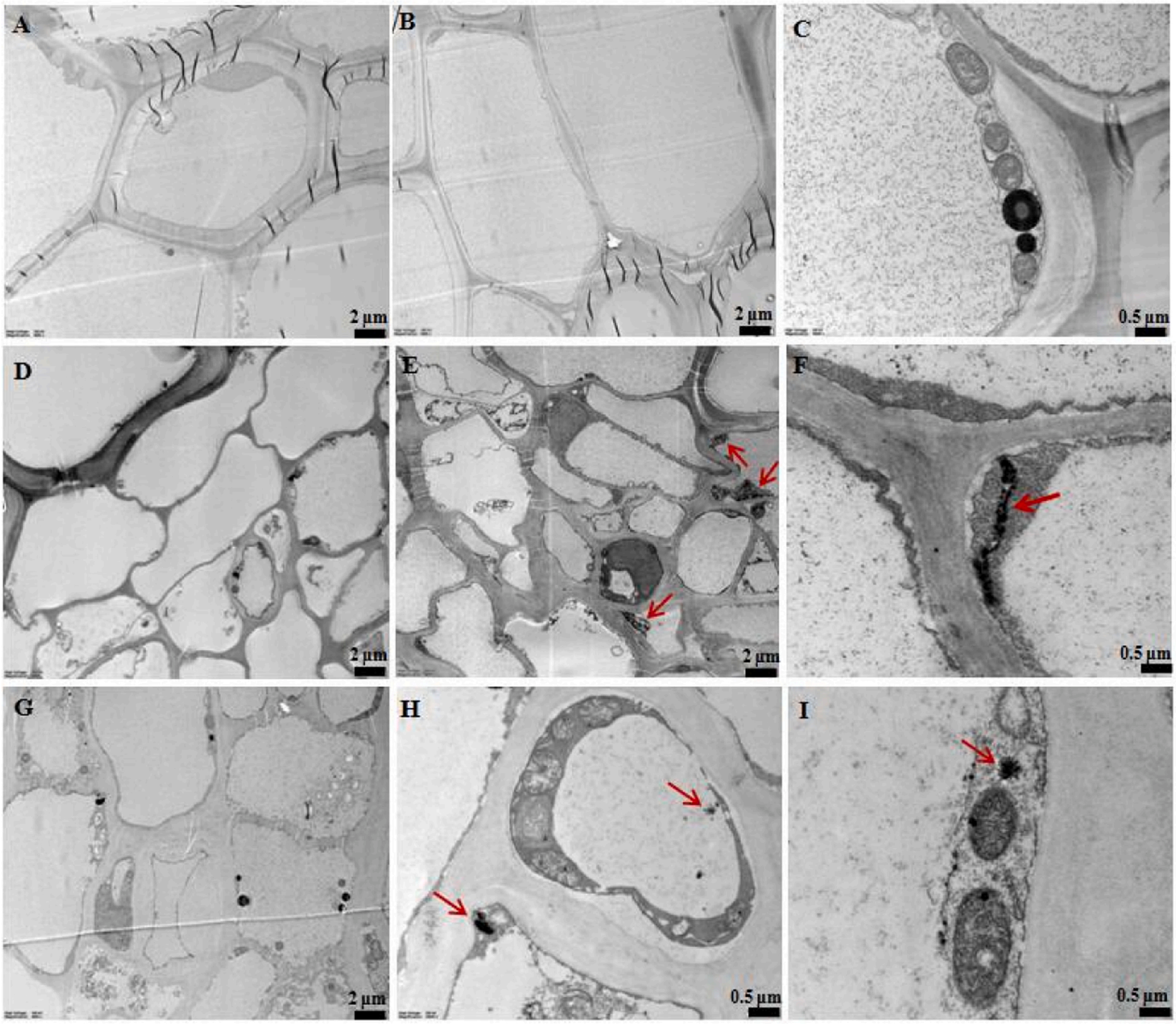

Fig. 2. TEM images of soybean roots upon the treatments of 0 (the fist line: A-C), 200 (the second line: D-F), and 500 (the third line: G-I) mg/kg of Cu NPs. It is obvious that after exposure with Cu NPs at 200 and 500 mg/kg the cells posed irregular morphology and included some particle aggregates.

observed (Fig. 2A-C). In contrast, upon the treatment of Cu NPs at the level of 200 mg/kg, the root cells exhibited a slight deformation with some wrinkles on the external surface (Fig. 2D and E). When the concentration of the Cu NPs was 500 mg/kg, the root cell structures were more seriously distorted, with much thicker cell walls in comparison with the control (Fig. 2G and H). It was furthermore obvious that particulates or aggregates were present in the cells upon the Cu NP treatments (Fig. 2E, F, H and I). The weight percentage of Cu in the root cells upon exposure to the Cu NPs at 200 mg/kg was much higher than that in the control (4.15% vs 0.83%, Fig. S3). The mechanisms underlying the internalization of MNPs with a size beyond the size exclusion limits of plant root cells remain unclear. The physical and chemical interactions between NPs and root cells could disrupt the cell wall organization and structure, leading to an enlargement of the cell wall pores, which further facilitates NPs to enter plant cells (Wan et al., 2019; Yang et al., 2017a, 2017b; Yuan et al., 2016; Larue et al., 2012). This might partially explain the higher Cu content upon exposure to the Cu NPs as compared to exposure to $CuSO_4$. Added to that, relative to the Cu NPs, $CuSO_4$ would exhibit a much more rapid release of Cu ions into soil pore water, resulting in a higher extent of complexation of the dissolved Cu ions with clay colloids and soil organic matters and therefore reducing the bioavailability of $CuSO_4$ to plants (Keller et al., 2017). In addition, the amount of the Cu-ions shedding from the Cu NPs may be enhanced due to the chemical interaction of the Cu NPs with root exudates (Rossi et al., 2019; Shang et al., 2019). This may also contribute to the higher amount of Cu taken up by the soybean roots upon exposure to Cu NPs than upon exposure to $CuSO_4$. However, these elucidations need further experimental confirmation.

In aerial parts of the soybeans (i.e., stems, leaves and beans), both the Cu NP and $CuSO_4$ treatments significantly increased the Cu contents in comparison with the control at both the flowering and maturation stages ($P < 0.05$) (Fig. 1C-F). Generally, the Cu contents in the aerial parts upon the Cu NP treatment were significantly higher than those upon the $CuSO_4$ treatment at an equivalent level ($P < 0.05$) (Fig. 1C-F), except the cases that the Cu contents in leaves and beans upon the treatment of 500 mg/kg Cu NPs were lower than those upon the $CuSO_4$ treatment (Fig. 1E and F). It is worth to note that there were significantly higher Cu contents in beans upon exposure to the Cu NP treatments at levels of 100 and 200 mg/kg than upon exposure to the $CuSO_4$ treatments (Fig. 1F). This indicates a probably higher risk to human health after intake of beans grown in soils to which Cu NPs ($\leq$ 200 mg/kg) have been added than after consumption of beans grown in soils to which an equivalent Cu salt has been added.

3.2. Growth responses of soybeans

The plant height and biomass was significantly improved when the Cu NPs and $CuSO_4$ were present at a level $\leq$ 200 mg Cu/kg, while the growth was significantly inhibited when the Cu NPs and $CuSO_4$ at 500 mg Cu/kg (Fig. 3). The growing trend of the soybean plants upon increasing concentrations of Cu NPs from 100 to 500 mg/kg was contrary to the Cu content taken up by the soybeans. A higher content of Cu taken up by the soybeans at a higher Cu NP concentration is likely to result in an excess of Cu in the soybeans, which could affect cell energy metabolism (Deng et al., 2020; Mustafa and Komatsu, 2016). The growth parameters (i.e., plant height and biomass) were significantly

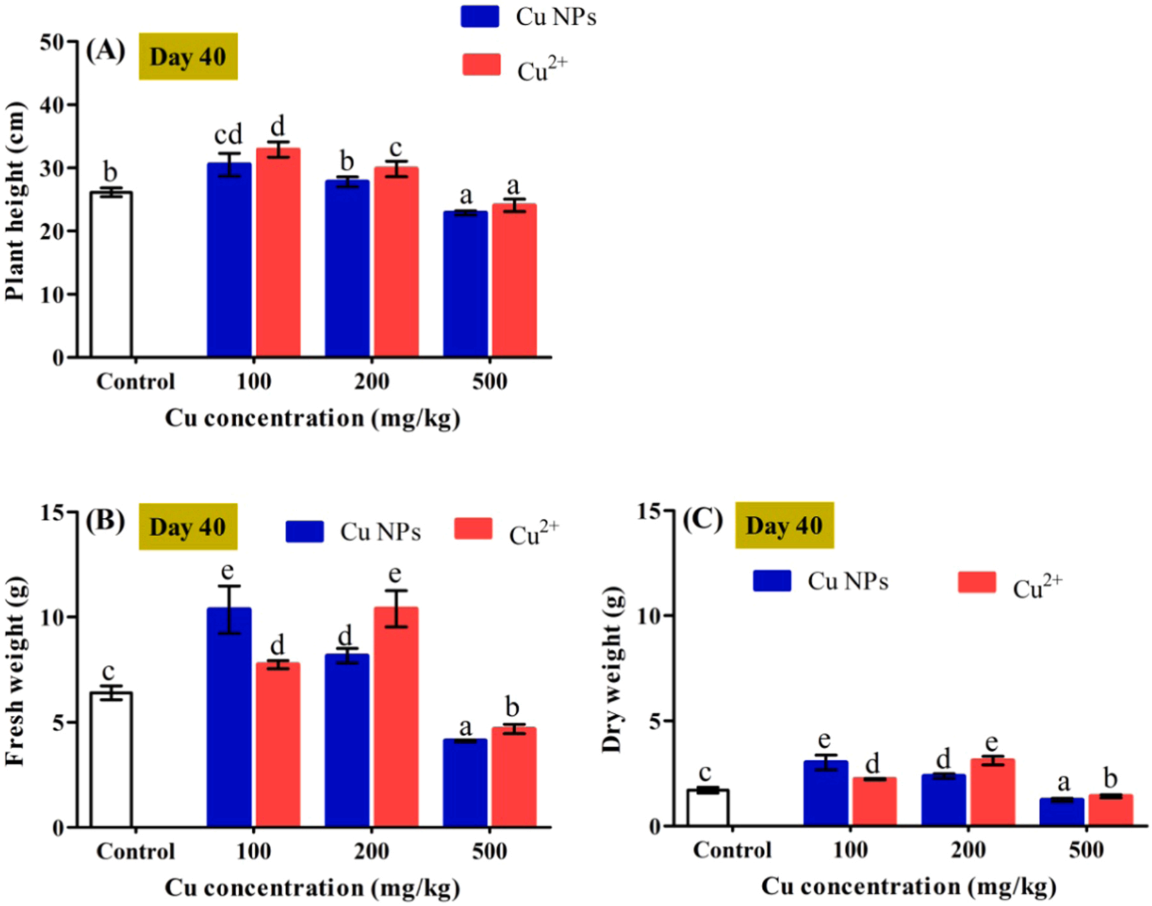

Fig. 3. Alterations of height (A), fresh weight (B), and dry weight (C) of the soybean plants after 40 d of incubation in soils amended with Cu NPs and Cu^{2+} at the concentration range from 0 to 500 mg/kg. Data are presented as mean ± SD ($n = 8$). Different letters indicate significant differences among different treatments as tested by one-way ANOVA and t-test ($P < 0.05$).

lower upon the Cu NP treatments at 200 and 500 mg Cu/kg than upon the $CuSO_4$ treatments. Similarly, Yusefi-Tanha et al. (2020) found that the seed production of soybeans upon CuO NP treatment at a concentration range from 200 to 500 mg/kg was significantly lower than in case of exposure to Cu^{2+} ions at an equal Cu concentration (Yusefi-Tanha et al., 2020). However, the authors did not compare the accumulation profile of Cu in the soybeans upon the nanoparticulate and ionic Cu treatments. According to our results on the Cu contents in the soybeans as depicted above, the relatively lower growth parameters might be attributed to the higher capacity of the Cu NPs than Cu^{2+} to be taken up by soybeans (Fig. 1). The total amounts of Cu taken up by the soybean plants after 40 d of cultivation were around 25 and 45 mg Cu/kg for the $CuSO_4$ and Cu NP treatments at the level of 200 Cu/kg and were around 35 and 66 mg Cu/kg upon the $CuSO_4$ and Cu NP treatments at the level of 500 Cu/kg, respectively (Fig. 1). Consequently, the higher Cu contents in the soybean plants upon exposure to the Cu NPs would probably result in a relative excess intake of Cu in the soybean plants. It is interesting to note that even though the Cu content taken up by the soybeans upon the Cu NP treatment at 200 mg/kg was higher than the Cu content upon the $CuSO_4$ treatment at the level of 500 mg/kg, the growth of the soybeans relative to the control was significantly inhibited upon the latter treatment rather than upon the former one. It has been demonstrated that Cu-based NPs have a higher potential to localize in plant vacuoles than Cu^{2+} (Yuan et al., 2016; Perreault et al., 2012). The sequestration of hazardous materials in vacuoles is an efficient way for plants to alleviate the stress resulting from the materials (Hall, 2002; Zenk, 1996). In addition, as important organic antidotes to metals, metallothionein and phytochelatins are more likely to form in plant root cells upon Cu-based NP treatment than that upon Cu salt treatment (Rawat et al., 2018; Fernandes and Henriques, 1991). These mechanisms might consequently result in the growth parameters of the soybean plants upon the $CuSO_4$ treatment at the level of 500 mg/kg being significantly lower than the growth parameters of the soybean plants upon the Cu NP treatment at the level of 200 mg/kg.

3.3. *Contents of photosynthetic pigments*

Photosynthesis is an important physiological and biochemical process in plants, the level of which to a large extent represents the adaptability of plants to external stresses (Niu et al., 2008). Chlorophyll and carotenoids in leaves are extremely important photosynthetic pigments, which are directly correlated with the intensity of photosynthesis. The content of Chl-*b* in the soybean leaves was more sensitive than the contents of Chl-*a* and carotenoids to the Cu NPs and $CuSO_4$ (Fig. 4). The contents of Chl-*a* and carotenoids were only significantly affected when the concentration of the Cu NPs was 500 mg/kg, as at that level the contents of Chl-*a* and carotenoids significantly decreased to 0.54 and 0.17 mg/g in comparison with the control (1.68 mg/g of Chl-*a* and 0.44 mg/g of carotenoids), respectively ($P < 0.05$) (Fig. 4A and B). The content of Chl-*b* increased significantly from 0.59 mg/g in the control to 0.76 and 0.71 mg/g upon the addition of the Cu NPs at 100 and 200 mg/kg and to 0.91 and 0.94 mg/g upon the addition of $CuSO_4$ at levels of 100 and 200 mg/kg, respectively ($P < 0.05$) (Fig. 4C). However, when the concentration of the Cu NPs reached 500 mg/kg, the content of the Chl-*b* was significantly decreased to 0.13 mg/g ($P < 0.05$) (Fig. 4C). Chl-*b* plays a critical role in improving the efficiency of light absorption and consequently increasing energy production in plants (Hassid and Putman, 1950). Therefore, the improvement of growth of the soybean plants upon the Cu NP and $CuSO_4$ treatments at levels of 100 and 200 mg/kg might be partially related to the increasing contents of Chl-*b*, as compared to the control. Our results suggest that the Chl-*b* content is a better marker than the Chl-*a* content for the effect of Cu NPs on soybeans. Similar to our results, there have been some studies

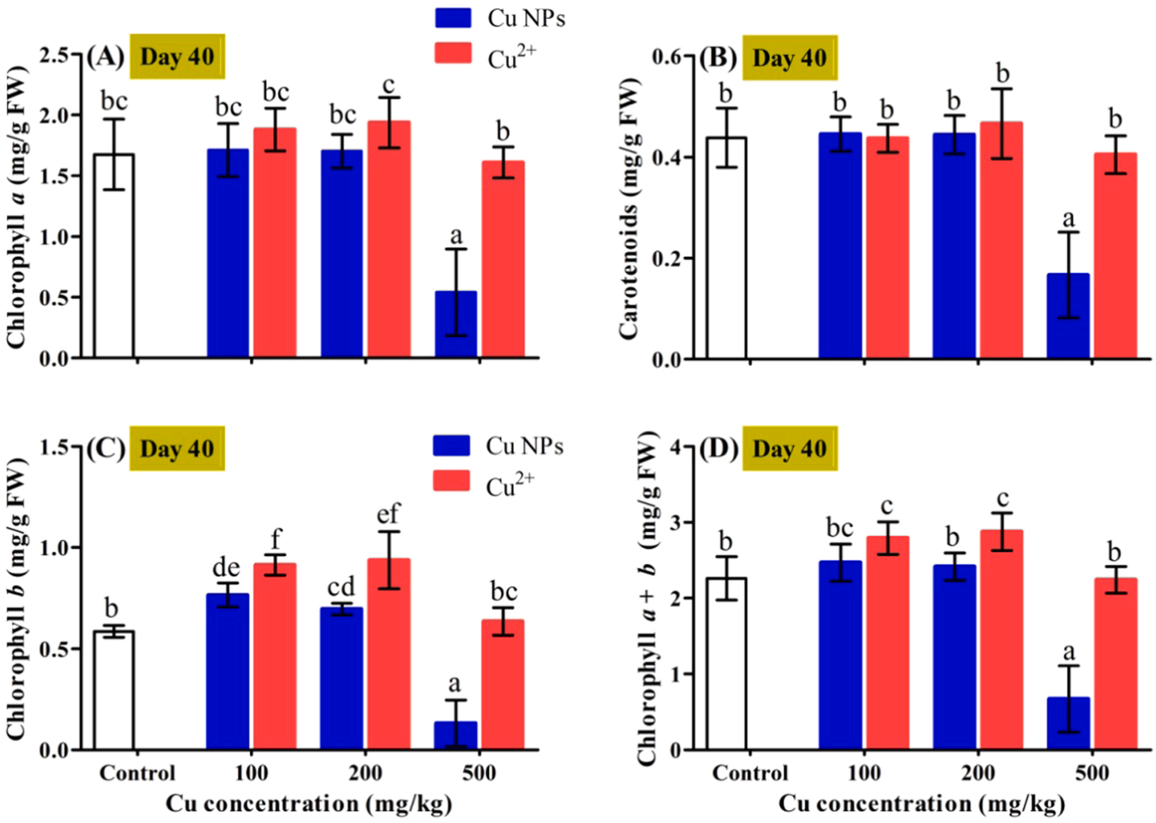

Fig. 4. Contents of chlorophyll *a* (A), carotenoids (B), chlorophyll *b* (C) and chlorophyll *a* + *b* (D) in the subapical leaves of the soybean plants after 40 d of incubation in the soils amended with Cu NPs and Cu^{2+} at the concentration range from 0 to 500 mg/kg. Data are presented as mean ± SD ($n = 4$). Different letters indicate significant differences among different treatments as tested by one-way ANOVA and *t*-test ($P < 0.05$).

demonstrating that the total chlorophyll content, as mainly constituted by Chl-*a* was not a sensitive marker for the MNP effects (Xu et al., 2019; Yuan et al., 2018; Zhao et al., 2013). In our study, the total contents of Chl-*a* and Chl-*b* were also non-significantly affected by the Cu NPs at levels of 100 and 200 mg/kg, as compared to the control ($P > 0.05$) (Fig. 4D). Therefore, specific effects of MNPs on different types of photosynthetic pigments are needed to be distinguished in order to better understand the response of plants to the MNPs.

3.4. Content of macroelements in soybeans

The contents of N, P and K in different parts of the soybean plants upon both the Cu NP and $CuSO_4$ treatments at the tested concentrations (0–500 mg Cu/kg) were determined (Fig. 5). The N content in the soybean roots was significantly decreased by 70%, 44% and 20% upon the Cu NP treatments and by 22%, 42%, and 33% upon the $CuSO_4$ treatments at 100, 200, and 500 mg/kg, respectively, in comparison with the control ($P < 0.05$) (Fig. 5A). Excess Cu could reduce the activities of enzymes involved in N transformation (e.g., nitrate reductase) in plants by binding with active sites of the enzymes (e.g., cysteine), and then inhibit the metabolism of N and consequently reduce the uptake of N by roots (Rui et al., 2018; Hippler et al., 2016; Xiong et al., 2006). The other possible mechanism involved in the reduction of N uptake by the roots upon exposure to Cu lies in an increase of breakdown of the enzymes for N transformations in plants due to the production of reactive oxygen species induced by excess Cu (Martins et al., 2012; Xiong et al., 2006). It is interesting to note that the N content in the roots upon the Cu NP treatment at 100 mg/kg was the lowest in comparison with the root N contents upon other treatments and the root N content upon the Cu NP treatment was significantly increased from 100 to 500 mg/kg ($P < 0.05$) (Fig. 5A). This might be due to the higher capability of Cu NPs to interfere N metabolism in plants at a relatively lower concentration as a result of less aggregation. On the other hand, all the Cu treatments did not significantly affect the N contents in the leaves ($P > 0.05$) (Fig. 5C). Similarly, Hao et al. (2016) found that the Fe-C nanotubes, FeCo-C nanotubes and C nanotubes at a concentration range of 10–300 mg/L significantly reduced the N contents in the rice roots in comparison with control, whereas they did not significantly affect the N contents in aerial parts of the rice tested. Further studies are needed to elucidate the mechanisms underlying the different effects of Cu compound treatments on the N contents in the roots and leaves.

Compared to the control, the P content in the roots was significantly decreased by exposure to Cu NPs, with a reduction of 15%, 24%, and 31% at levels of 100, 200, and 500 mg/kg, respectively ($P < 0.05$) (Fig. 5D). However, upon the $CuSO_4$ treatment the P content in the roots was only significantly decreased at the level of 500 mg/kg by 28%, as compared to the control ($P < 0.05$) (Fig. 5D). In fact, Cu-based NPs have the potential to reduce the uptake of P in plant roots by physically blocking the P transporters and/or by limiting the bioavailability of P via forming Cu phosphates compounds (Zuverza-Mena et al., 2015). Even though the P content in the roots was significantly reduced by the Cu NPs, the P contents in aerial parts (stems and leaves) of the soybeans upon the Cu NP treatments were similar to the control (Fig. 5E and F). This implies that the transport efficiency of P in the soybeans was improved upon the Cu NP treatment.

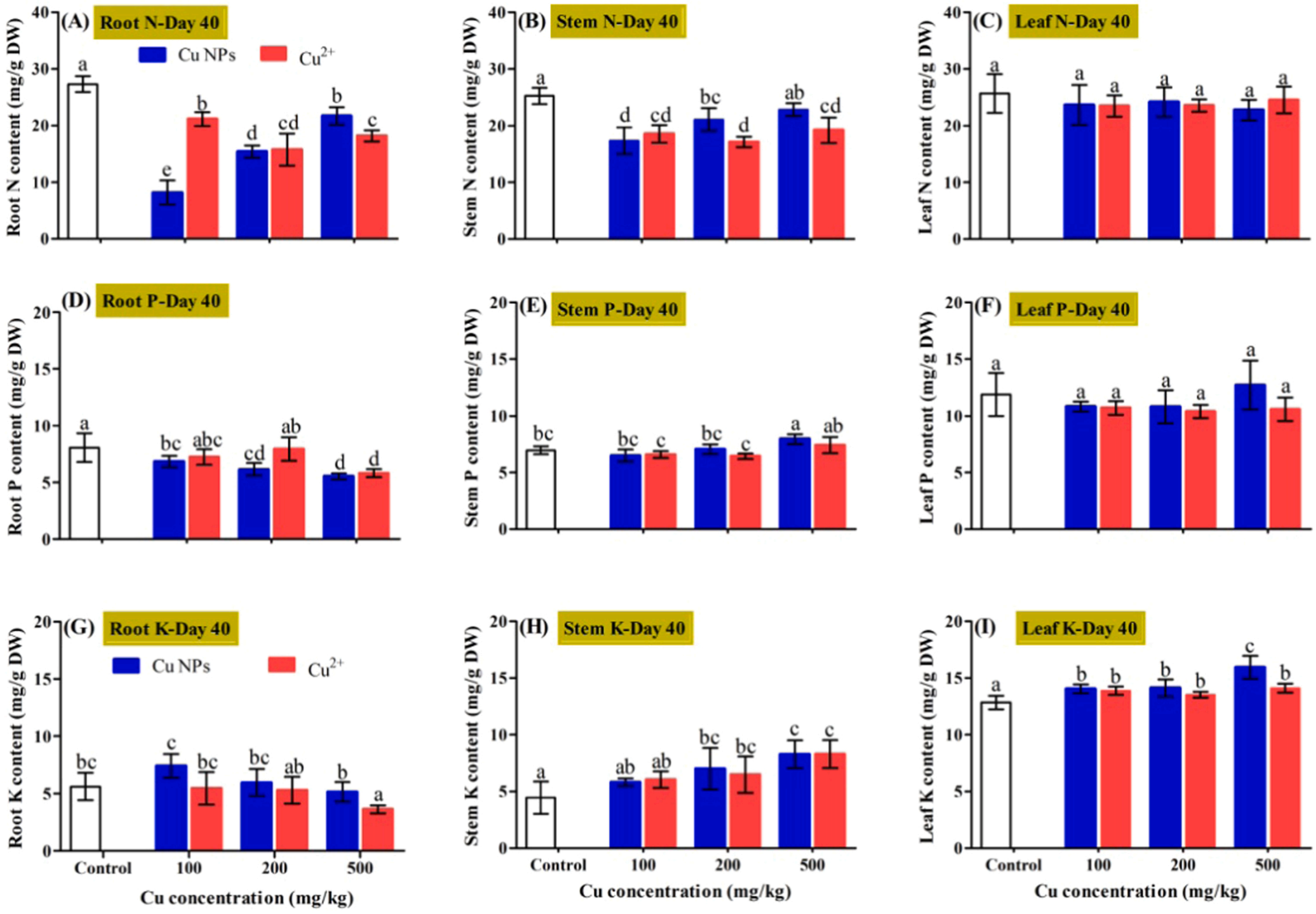

Fig. 5. Contents of N, P and K in different parts-roots (A, D, G), stems (B, E, H) and leaves (C, F, I)-of the soybean plants on Day 40 at 0, 100, 200, 500 mg/kg of soil treatments with Cu NPs and Cu^{2+}. Data are presented as mean ± SD ($n = 4$). Different letters indicate significant differences among different treatments as tested by one-way ANOVA and *t*-test ($P < 0.05$).

The K content in the soybean roots was not significantly affected by the two Cu compound treatments, except the case in which the $CuSO_4$ at 500 mg/kg significantly reduced the K content in roots in comparison with the control (Fig. 5G). However, in aerial parts of the soybean plants, the K content was increased by the two Cu compounds (Fig. 5H and I). Especially in the soybean leaves, all the Cu treatments significantly increased the K contents in the leaves, as compared to the control ($P < 0.05$) (Fig. 5I). Currently, the reduction, promotion and even non-significant effects of MNPs on the accumulation of K in plants have all been reported (Abbas et al., 2020; Deng et al., 2020; Dimkpa et al., 2019; Peralta-Videa et al., 2014). The precise mechanisms for the alterations of MNPs on the K accumulation in plants are still unknown. The increasing K content in aerial parts of the soybean plants might be related to the activating effect of Cu on the HKT1 transporters (Schachtman and Schroeder, 1994), which therefore improved the transport of K from roots to aerial parts of the soybeans.

4. Conclusion

This study found that the Cu NPs could induce more Cu accumulation in the soybeans than $CuSO_4$. Particulate aggregates was observed in the root cells with deformation upon the Cu NP treatment. The Cu NPs at 100 and 200 mg/kg could improve the soybean height and biomass in comparison with the control, yet significantly inhibited the growth at 500 mg/kg. Relative to the contents of Chl-*a* and carotenoids in the soybean leaves, the Chl-*b* content was more sensitive to the two Cu compounds. Both the CuNPs and $CuSO_4$ at 100 and 200 mg/kg significantly increased the *Chl*-b content in leaves, although the increasing effect induced by the CuNPs was lower than $CuSO_4$. The contents of N, P and K in the soybean plants exposed to Cu NPs at 100–500 mg/kg were altered, with reductions of N and P in roots and an increase of K in leaves. Our results imply that caution with regard to the application of Cu NPs in agriculture is warranted, as more Cu would potentially be taken up by people after dietary consumption of the healthy-looking soybeans with altered nutritious quality planted in soils to which Cu-based NPs have been added.

CRediT authorship contribution statement

Yinlong Xiao: Conceptualization, Methodology, Writing, Funding acquisition**. Jun Ma**: Investigation, Writing – original draft. **Junren Xian**: Supervision, Resources. **Willie J. G. M. Peijnenburg**: Reviewing, Validation. **Ying Du**: Investigation, Funding acquisition. **Dong Tian**: Formal analysis, Resources. **Hong Xiao**: Investigation. **Ouping Deng**: Resources, Writing – review & editing. **Lihua Tu**: Supervision, Reviewing. **Yan He:** Resources, Project administration. **Ling Luo**: Review & Editing.

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.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (Grant No. 21906109) and by the National College Students Innovation and Entrepreneurship Training Programs (Grant No. 201910626131).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.113088.

References

Abbas, Q., Yousaf, B., Ullah, H., Ali, M.U., Zia-Ur-Rehman, M., Rizwan, M., Rinklebe, J., 2020. Biochar-induced immobilization and transformation of silver-nanoparticles affect growth, intracellular-radicles generation and nutrients assimilation by reducing oxidative stress in maize. J. Hazard. Mater. 390, 121976.

Achari, G.A., Kowshik, M., 2018. Recent developments on nanotechnology in agriculture: plant mineral nutrition, health, and interactions with soil microflora. J. Agric. Food Chem. 66, 8647–8661.

Anjum, N.A., Adam, V., Kizek, R., Duarte, A.C., Pereira, E., Iqbal, M., Lukatkin, A.S., Ahmad, I., 2015a. Nanoscale copper in the soil-plant system-toxicity and underlying potential mechanisms. Environ. Res. 138, 306–325.

Anjum, N.A., Singh, H.P., Khan, M.I.R., Masood, A., Per, T.S., Negi, A., Batish, D.R., Khan, N.A., Duarte, A.C., Pereira, E., Ahmad, I., 2015b. Too much is bad-an appraisal of phytotoxicity of elevated plant-beneficial heavy metal ions. Environ. Sci. Pollut. Res. 22, 3361–3382.

Bayat, N., Rajapakse, K., Marinsek-Logar, R., Drobne, D., Cristoba, S., 2014. The effects of engineered nanoparticles on the cellular structure and growth of *Saccharomyces cerevisiae*. Nanotoxicology 8, 363–374.

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

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

Du, W., Gardea-Torresdey, J.L., Ji, R., Yin, Y., Zhu, J.G., Peralta-Videa, J.R., Guo, H.Y., 2015. Physiological and biochemical changes imposed by CeO_2 nanoparticles on wheat: a life cycle field study. Environ. Sci. Technol. 49, 11884–11893.

Fernandes, J., Henriques, F., 1991. Biochemical, physiological, and structural effects of excess copper in plants. Bot. Rev. 57, 246–273.

Gardea-Torresdey, J.L., Rico, C.M., White, J.C., 2014. Trophic transfer, transformation, and impact of engineered nanomaterials in terrestrial environments. Environ. Sci. Technol. 48, 2526–2540.

Grimshaw, H.M., Allen, S.E., Parkinson, J.A., 1989. Nutrient elements. In: Allen, S.E. (Ed.), Chemical Analysis of Ecological Material. Blackwell Scientific, Oxford, pp. 81–159.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot. 53, 1–11.

Hao, Y., Yu, F., Lv, R., Ma, C., Zhang, Z., Rui, Y., Liu, L., Cao, W., Xing, B., 2016. Carbon nanotubes filled with different ferromagnetic alloys affect the growth and development of rice seedlings by changing the C:N ratio and plant hormones concentrations. Plos One 11, e0157264.

Harris, A.T., Bali, R., 2008. On the formation and extent of uptake of silver nanoparticles by live plants. J. Nanopart. Res. 10, 691–695.

Hassid, W., Putman, E., 1950. Transformation of sugars in plants. Annu. Rev. Plant Physiol. 1, 109–124.

Hippler, F.W.R., Cipriano, D.O., Boaretto, R.M., Quaggio, J.A., Gaziola, S.A., Azevedo, R. A., Mattos-Jr, D., 2016. Citrus rootstocks regulate the nutritional status and antioxidant system of trees under copper stress. Environ. Exp. Bot. 130, 42–52.

Keller, A.A., Lazareva, A., 2014. Predicted releases of engineered nanomaterials: from global to regional to local. Environ. Sci. Technol. Lett. 1, 65–70.

Keller, A.A., McFerran, S., Lazareva, A., Suh, S., 2013. Global life cycle releases of engineered nanomaterials. J. Nanopart. Res. 15, 1–17.

Keller, A.A., Adeleye, A.S., Conway, J.R., Garner, K.L., Zhao, L., Cherr, G.N., Hong, J., Gardea-Torresdey, J.L., Godwin, H.A., Hanna, S., Ji, Z., Kaweeteerawat, C., Lin, S., Lenihan, H.S., Miller, R.J., Nel, A.E., Peralta-Videa, J.R., Walker, S.L., Taylor, A.A., Torres-Duarte, C., Zink, J.I., Zuverza-Mena, N., 2017. Comparative environmental fate and toxicity of copper nanomaterials. NanoImpact 7, 28–40.

Korshunova, Y.O., Eide, D., Clark, W.G., Guerinot, M.L., Pakrasi, H.B., 1999. IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Mol. Biol. 40, 37–44.

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.

Larue, C., Laurette, J., Herlin-Boime, N., Khodja, H., Fayard, B., Flank, A.M., Brisset, F., Carriere, M., 2012. Accumulation, translocation and impact of TiO_2 nanoparticles in wheat (*Triticum aestivum* spp.): influence of diameter and crystal phase. Sci. Total Environ. 431, 197–208.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzym. 148, 350–382.

Lopez-Moreno, M.L., de la Rosa, G., Hernandez-Viezcas, J.A., Castillo-Michel, H., Botez, C.E., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2010. Evidence of the differential biotransformation and genotoxicity of ZnO and CeO_2 nanoparticles on soybean (*Glycine max*) plants. Environ. Sci. Technol. 44, 7315–7320.

Martins, V., Hanana, M., Blumwald, E., Gerós, H., 2012. Copper transport and compartmentation in grape cells. Plant Cell Physiol. 53, 1866–1880.

Mary, G., Bajpai, S.K., Chand, N., 2009. Copper (II) ions and copper nanoparticles-loaded chemically modified cotton cellulose fibers with fair antibacterial properties. J. Appl. Polym. Sci. 113, 757–766.

Mustafa, G., Komatsu, S., 2016. Toxicity of heavy metals and metal-containing nanoparticles on plants. Biochim. Biophys. Acta 1864, 932–944.

Nekrasova, G.F., Ushakova, O.S., Ermakov, A.E., Uimin, M.A., Byzov, I.V., 2011. Effects of copper (II) ions and copper oxide nanoparticles on *Elodea densa* planch. Russ. J. Ecol. 42, 458–463.

Niu, G., Rodriguez, D.S., Mackay, W., 2008. Growth and physiological responses to drought stress in four oleander clones. J. Am. Soc. Hortic. Sci. 133, 188–196.

Peijnenburg, W., Praetorius, A., Scott-Fordsmand, J., Cornelis, G., 2016. Fate assessment of engineered nanoparticles in solids dominated media-current insights and the way forward. Environ. Pollut. 218, 1365–1369.

Peralta-Videa, J.R., Hernandez-Viezcas, J.A., Zhao, L., Diaz, B.C., Ge, Y., Priester, J.H., Holden, P.A., Gardea-Torresdey, J.L., 2014. Cerium dioxide and zinc oxide nanoparticles alter the nutritional value of soil cultivated soybean plants. Plant Physiol. Biochem. 80, 128–135.

Perreault, F., Oukarroum, A., Melegari, S.P., Matias, W.G., Popovic, R., 2012. Polymer coating of copper oxide nanoparticles increases nanoparticles uptake and toxicity in the green alga *Chlamydomonas reinhardtii*. Chemosphere 87, 1388–1394.

Priester, J.H., Ge, Y., Mielke, R.E., Horst, A., Moritz, S., Espinosa, K., Gelb, J., Walker, S., Nisbet, R., An, Y., Schimel, J., Palmer, R., Hernandez-Viezcas, J., Zhao, L., Gardea-Torresdey, J., Holden, P., 2012. Soybean susceptibility to manufactured nanomaterials with evidence for food quality and soil fertility interruption. Proc. Natl. Acad. Sci. U SA 109, 2451–2456.

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.

Rossi, L., Sharifan, H., Zhang, W., Schwab, A.P., Ma, X., 2018. Mutual effects and in planta accumulation of co-existing cerium oxide nanoparticles and cadmium in hydroponically grown soybean (*Glycine max* (L.) Merr. Environ. Sci. Nano 5, 150–157.

Rossi, L., Bagheri, M., Zhang, W., Chen, Z., Burken, J., Ma, X., 2019. Using artificial neural network to investigate physiological changes and cerium oxide nanoparticles and cadmium uptake by *Brassica napus* plants. Environ. Pollut. 246, 381–389.

Rui, M., Ma, C., White, J., Hao, Y., Wang, Y., Tang, X., Yang, J., Jiang, F., Ali, A., Rui, Y., Cao, W., Chen, G., Xing, B., 2018. Metal oxide nanoparticles alter peanut (*Arachis hypogaea* L.) physiological response and reduce nutritional quality: a life cycle study. Environ. Sci. Nano 5, 2088–2102.

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.

Schwabe, F., Schulin, R., Limbach, L.K., Stark, W., Burge, D., 2013. Influence of two types of organic matter on interaction of CeO_2 nanoparticles with plants in hydroponic culture. Chemosphere 91, 512–520.

Shang, H., Guo, H., Ma, C., Li, C., Chefetz, B., Polubesova, T., Xing, B., 2019. Maize (*Zea mays* L.) root exudates modify the surface chemistry of CuO nanoparticles: altered aggregation, dissolution and toxicity. Sci. Total Environ. 690, 502–510.

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.

Singh, A., Singh, N.B., Hussain, I., Singh, H., 2017. Effect of biologically synthesized copper oxide nanoparticles on metabolism and antioxidant activity to the crop plants *Solanum lycopersicum* and *Brassica oleracea* var. botrytis. J. Biotechnol. 262, 11–27.

Thomas, R.L., Sheard, R.W., Moyer, J.R., 1967. Comparison of conventional and automated procedures for nitrogen phosphorus and potassium analysis of plant material using a single digestion. Agron. J. 59, 240–243.

Tisdall, J.M., Oades, J.M., 1979. Stabilization of soil aggregates by the root systems of ryegrass. Aust. J. Soil Res. 17, 429–441.

Vance, M.E., Kuiken, T., Vejerano, E.P., Mcginnis, S.P., Hochella Jr, M.F., Rejeski, D., Hull, M.S., 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein J. Nanotechnol. 6, 1769–1780.

Wan, J.P., Wang, R.T., Wang, R.L., Ju, Q., Wang, Y.B., Xu, J., 2019. Comparative physiological and transcriptomic analyses reveal the toxic effects of ZnO nanoparticles on plant growth. Environ. Sci. Technol. 53, 4235–4244.

Xiong, Z.T., Liu, C., Geng, B., 2006. Phytotoxic effects of copper on nitrogen metabolism and plant growth in *Brassica pekinensis* Rupr. Ecotoxicol. Environ. Saf. 64, 273–280.

Xu, M.L., Zhu, Y.G., Gu, K.H., Zhu, J.G., Yin, Y., Ji, R., Du, W.C., Guo, H.Y., 2019. Transcriptome reveals the rice response to elevated free air CO_2 concentration and TiO_2 nanoparticles. Environ. Sci. Technol. 53, 11714–11724.

Yan, D., Zhao, Y., Lu, A., Wang, S., Xu, D., Zhang, P., 2013. Effects of accompanying anions on cesium retention and translocation via droplets on soybean leaves. J. Environ. Radioact. 126, 232–238.

Yang, J., Jiang, F., Ma, C., Rui, Y., Rui, M., Adeel, M., Cao, W., Xing, B., 2018. Alteration of crop yield and quality of wheat upon exposure to silver nanoparticles in a life cycle study. J. Agric. Food Chem. 66, 2589–2597.

Yang, X., Pan, H., Wang, P., Zhao, F.J., 2017b. Particle-specific toxicity and bioavailability of cerium oxide (CeO_2) nanoparticles to *Arabidopsis thaliana*. J. Hazard. Mater. 322, 292–300.

Yang, Y., Christakos, G., Guo, M., Xiao, L., Huang, W., 2017a. Space-time quantitative source apportionment of soil heavy metal concentration increments. Environ. Pollut. 223, 560–566.

Yruela, I., 2005. Copper in plants. Braz. J. Plant Physiol. 17, 145–156.

Yuan, J., He, A., Huang, S., Hua, J., Sheng, G.D., 2016. Internalization and phytotoxic effects of CuO nanoparticles in *Arabidopsis thaliana* as revealed by fatty acid profiles. Environ. Sci. Technol. 50, 10437–10447.

Yuan, L., Richardson, C.J., Ho, M., Willis, C.W., Colman, B.P., Wiesner, M.R., 2018. Stress responses of aquatic plants to silver nanoparticles. Environ. Sci. Technol. 52, 2558–2565.

Yusefi-Tanha, E., Fallah, S., Rostamnejadi, A., Pokhrel, L.R., 2020. Particle size and concentration dependent toxicity of copper oxide nanoparticles (CuONPs) on seed yield and antioxidant defense system in soil grown soybean (*Glycine max* cv. Kowsar). Sci. Total Environ. 715, 1–13.

Zenk, M.H., 1996. Heavy metal detoxification in higher plants-a review. Gene 179, 21–30.

Zhang, Z.Y., Ke, M.J., Qu, Q., Peijnenburg, W.J.G.M., Lu, T., Zhang, Q., Ye, Y.Z., Xu, P.F., Du, B.B., Sun, L.W., Qian, H.F., 2018. Impact of copper nanoparticles and ionic copper exposure on wheat (*Triticum aestivum* L.) root morphology and antioxidant response. Environ. Pollut. 239, 689–697.

Zhao, L., Sun, Y., Hernandez-Viezcas, J.A., Servin, A.D., Hong, J., Niu, G., Peralta-Videa, J.R., Duarte-Gardea, M., Gardea-Torresdey, J.L., 2013. Influence of CeO_2 and ZnO nanoparticles on cucumber physiological markers and bioaccumulation of Ce and Zn: a life cycle study. J. Agric. Food Chem. 61, 11945–11951.

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.