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Effects of copper oxide nanoparticles on growth of lettuce (*Lactuca sativa* L.) seedlings and possible implications of nitric oxide in their antioxidative defense

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Abstract Copper oxide nanoparticles (CuO NPs) have been extensively explored for use in agriculture. Previous studies have indicated that application of CuO NPs might be promising for development and conservation of plants, pest control, and for the recovery of degraded soils. However, depending on the applied concentration copper can cause phytotoxic effects. In this work, biosynthesized CuO NPs (using green tea extract) were evaluated on their effects on lettuce (*Lactuca sativa* L.) seedling growth, which were exposed at concentrations ranged between 0.2 and 300 μg mL^{-1}. From the biosynthesized were obtained ultra-small CuO NPs (~ 6.6 nm), with high stability in aqueous suspension. Toxicity bioassays have shown that at low concentrations (up to 40 μg mL^{-1}), CuO NPs did not affect or even enhanced the seed germination. At higher concentrations (higher than 40 μg mL^{-1}), inhibition of seed germination and radicle growth ranging from 35 to 75% was observed. With the increase of CuO NPs concentrations, nitrite and S-nitrosothiols levels in radicles increased, whereas superoxide dismutase and total antioxidant activities decreased. The nitrite and S-nitrosothiols levels in lettuce radicles showed a direct dose response to CuO NP application, which may indicate nitric oxide-dependent signaling pathways in the plant responses. Therefore, the results demonstrated that at low concentrations (≤ 20 μg mL^{-1}) of CuO NPs, beneficial effects are obtained from seedlings, enhancing plant growth, and the involvement of nitric oxide signaling in the phytotoxic effects induced by high concentration of this formulation.

Keywords Green tea · Metallic nanoparticles · Nanophytotoxicity · Plants · Seed · S-nitrosothiols

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Introduction

Global population increases, and strategies for a sustainable agriculture are necessary for food safety and security. Moreover, there is a strong interest in food quality and preservation, as well as in decreasing losses during production and distribution. The rise of global population, climate change, loss of productivity force, and

intense urbanization is a huge challenge to be overcome for better food production and distribution (FAO, I., WFP, W., and UNICEF 2019). NPs may pose a promising strategy with positive impacts on plant growth and physiology, seed germination, and protection against pathogens (Seabra et al. 2014). NPs also help plants tolerate biotic and abiotic stresses, soil remediation, and plant growth (Liu et al. 2016; Xiong et al. 2017; Rajput et al. 2018). Thus, they could contribute to improve food security.

The main desirable applications of nanotechnology are (i) decreasing the amount of hazardous chemical substances in the environment, (ii) decreasing losses of nutrients during fertilization, and (iii) increasing productivity by pest management and nutrients (Prasad et al. 2017). Besides that, most vegetables are very sensible to post-harvest practices (transport, storage, and the retail shelf) (Managa et al. 2018), and NPs have also been used as an alternative to face this problem (López-Vargas et al. 2018). Nevertheless, studies aiming the assessment of NPs occupational exposition, adverse effects, biosafety, and toxicology are still scarce. These kinds of assessment should be considered to mitigate undesirable impacts on agrosystems and human health (Iavicoli et al. 2017), but they are beyond the scope of this present study.

CuO NPs have been recently applied in agriculture as pesticides, herbicides, fertilizers (Spielman-Sun et al. 2018; Laughton et al. 2019; Wang et al. 2019), and bactericides (Zain et al. 2014; Kumar et al. 2015; Acharyulu et al. 2014). Recently, some authors presented results about CuO applications on wheat (*Triticum aestivum* L.) (Zhou et al. 2011; Gao et al. 2018), rice (*Oryza sativa* L.) (Peng et al. 2015; Peng et al. 2017), maize (*Zea mays* L.) (Wang et al. 2012; Adhikari et al. 2016), spinach (*Spinacia oleracea*) (Singh and Kumar 2016), transgenic cotton (Le Van et al. 2016), and lettuce (*Lactuca sativa* L.) (Hong et al. 2015; Liu et al. 2016; Laughton et al. 2019; Wang et al. 2019). In this present study, CuO NPs were applied on lettuce seedlings.

Worldwide, lettuce is one of the most common edible leaf vegetables. Lettuce has high water content (95%), fibers, vitamins (A, B1, B2, B3, B9, C, and E), beta-carotenes, phenolic compounds, minerals, carotenoids, and low caloric content (Kim et al. 2016; Shams et al. 2019). In vitro and in vivo studies reported health benefits due to lettuce consumption, such as the decrease of cholesterol (Lee et al. 2009), inflammatory processes (Pepe et al. 2015), diabetes (Cheng et al. 2014), and other conditions.

CuO NPs are commonly prepared by traditional synthesis using toxic reagents, organic solvents, several synthetic routes, controlled atmosphere, high temperatures, and toxic byproducts (Gawande et al. 2016). In contrast, biogenic or biological synthesis of NPs is considered eco-friendly, cost effective, and non-toxic (Rolim et al. 2019a). Biogenic synthesis is important for a future perspective on nanomaterials, mainly concerning issues related to safety and environment. Some biological agents to synthesize NPs have been instigated, such as enzymes (Kharissova et al. 2013), vitamins (Shao et al. 2018), algae (Bakir et al. 2018), bacteria (Presentato et al. 2018), fungi (Vijayanandan and Balakrishnan 2018), and also plant extract (Pirtarighat et al. 2019; Rolim et al. 2019a). In addition, to be easily performed and occur at room temperature and atmospheric pressure, the biological agent reduces metal ions to a smaller oxidation state forming the NPs and also acts as capping agent, protecting the NPs of oxidation and degradation (Nasrollahzadeh et al. 2019). Among the biological agents that can be used for this synthesis of NPs, tea extracts are rich in polyphenols and antioxidant agents. In particular, green tea extract (*Camellia sinensis*) is rich in catechin, a polyphenolic antioxidant agent found in plants as a secondary metabolite (Sharangi 2009; Nasrollahzadeh et al. 2019; Rolim et al. 2019a).

CuO NPs can be used as nutrients for plants but can have phytotoxicity depending on its concentration, size, surface coating, administration method, and time of exposure (Liu et al. 2016). Most of the previous studies have focused on the effect of CuO NPs on plants through physiological and morphological approaches. Phytotoxicity or benefits of NPs for plants can be evaluated by measuring seed germination, growth, and some signaling responses to oxidative stress induced by reactive oxygen species (ROS) and associated antioxidant mechanisms, which include enzymes such as catalase (CAT), superoxide dismutase (SOD), and peroxidases (POD) like ascorbate peroxidase (APX) (López-Vargas et al. 2018; Shams et al. 2019).

To better understand the mechanisms and beneficial effects of NPs on plants, reactive nitrogen species (RNS) could also be investigated. Nitric oxide (NO) is a small, highly diffusible gas and a very multitasked bioactive molecule (Nabi et al. 2019). Previous studies demonstrated that NO is an important player in the

modulation of gene expression and protein activity, interacting with ROS and controlling hormone actions (Seabra and Oliveira 2016). The NO production may play a role in signaling and stress acclimation to protect the plant against potentially toxic elements (Terrón-Camero et al. 2019). These metals enter in plant cells via specific transporters—for example, cation transporters minerals. Once they accumulate in a plant, they may cause toxicity through competition for cation absorption in the roots, interaction with thiolate proteins, and ROS generation. In particular, CuO NPs may be accumulated in the roots by both Cu and Cu-sulfur complexes (Dimkpa et al. 2013).

Therefore, this study aimed to (i) synthesize CuO NPs using a commercial green tea extract as a reducing and capping agent, (ii) characterize the obtained CuO NPs, (iii) evaluate CuO NPs dose-response on seed germination, and (iv) examine the ROS of seedlings response using the determination of antioxidant activity (SOD, POD, CAT, APX, and also RNS), by the determination of nitrite (NO_2^-) and S-nitrosothiol (RSNO) levels, to disclose the oxidative defense of lettuce seedlings under CuO NPs stress.

Materials and methods

Reagents

Copper chloride II ($CuCl_2$), *N*-ethylmaleimide (NEM), potassium iodide (KI), 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), sodium nitrite ($NaNO_2$), phosphate buffer (PBS, 7.4), ethylenediamine tetraacetic acid (EDTA), hydrogen peroxide (H_2O_2), poly(vinylpolypyrrolidone) (PVP), pyrogallol, potassium phosphate buffer, ascorbic acid methionine, riboflavin, and nitroblue tetrazolium chloride were analytical grade (Sigma-Aldrich, Missouri, USA). Sulfuric acid (H_2SO_4) was purchased from LabSynth (Diadema, SP, Brazil). Green tea powder (*Camellia sinensis*) was obtained from Sumioka Shokuhin Kabushikikaisha (Hiraguti, Japan).

Green synthesis of CuO nanoparticles

An aqueous solution of $CuCl_2$ (93.0 mmol L^{-1}) was added dropwise (25 drops per 10 s) into a suspension of green tea extract (2.5 mg mL^{-1}), prepared with ultrapure water, heated at 90 °C, and vacuum filtered with a qualitative filter. The volumetric proportion of $CuCl_2$ and green tea extract was 1 to 2, respectively. The final suspension pH was adjusted to 5.4 using NaOH (1 mol L^{-1}), then the mixture was homogenized for 15 min by magnetic stirring. The final mixture was centrifuged at 1731. 5×*g* and washed twice using ultrapure water. This process led to the formation of a black precipitate of CuO NPs, which were freeze-dried and then stored in a desiccator protected from light (Rolim et al. 2019a and 2019b).

Characterization of green tea-synthesized CuO nanoparticles

The hydrodynamic size, polydispersity index (PDI), and zeta potential values of green tea-synthesized CuO NPs were analyzed by dynamic light scattering (DLS, Zetasizer Nano ZS, Malvern Instruments Co, UK) (Rolim et al. 2019a and b). The morphology of CuO NPs was obtained by transmission electron microscopy (TEM) at 80 kV (Carl Zeiss 120 TEM, Zeiss International, Oberkochen, Germany) (Rolim et al. 2019b). The NP chemical surface characterization was performed using an X-ray photoelectron spectroscopy (XPS, K-alfa, Thermo Fisher Scientific, UK) with a monochromatic argonium source and a charge neutralizer (Rolim et al. 2019b; Liu et al. 2014b).

Bioassays with *Lactuca sativa*

Lettuce *(L. sativa* L. cv Hanson of the family Asteraceae) seeds were used in the bioassays to assess the phytotoxicity of CuO NPs. Green tea-synthesized CuO NPs were dispersed in the growing solution (OECD recommended ISO test medium) consisting of (g L^{-1}): 0.294 $CaCl_2{\cdot}2H_2O$, 0.123 $MgSO_4{\cdot}7H_2O$, 0.065 $NaHCO_3$, 0.006 KCl (OECD 2004). The CuO NPs suspension was sonicated for 60 min. The bioassays were performed at different CuO NPs concentrations (0.2, 2, 20, 40, 80, 150, and 300 μg mL^{-1}), according to the methodology proposed by Belo (2011) with some modifications. The assays were carried out on glass Petri dish (90 nm diameter) with qualitative filter paper inside and moistened with 3 mL of CuO NPs suspension of each concentration. No disinfection treatment was applied to the seeds before the bioassay test to avoid a phytotoxic effect of the disinfection agent (Salazar-Mercado et al. 2019). Six seeds were centrally and uniformly (in-line) placed on each Petri dish. Subsequently, the Petri dishes were covered and sealed with

parafilm to avoid moisture losses by evaporation. The Petri dishes were placed in an upright position in an incubator (Solab, Brazil) at 22 ± 2 °C for 5 days in dark. ISO water growing medium alone (OECD 2004) was used as positive control. The experiments were performed in six replicates. After the 5-day period, the number of germinated seeds was visually assessed and the root length of each seedling was measured using a digital caliper (Mtx, 150 mm). Then, the relative percentage of seed germination (%RSG), relative percentage of root growth (%RRG), and the germination index (GI) were calculated.

To calculate the %RSG for each plate, Eq. 1 was used, where $\bar{\bar{S}}g_s$ is the arithmetic mean of the number of germinated seeds of the treated sample; $\bar{\bar{S}}g_c$ is the arithmetic mean of the number of germinated seeds in the control:

$$\%RSG = \frac{\bar{\bar{S}}g_s}{\bar{\bar{S}}g_c} \times 100\% \quad (1)$$

To calculate the %RRG for each plate, Eq. 2 was used, where $\bar{L}r_s$ is the arithmetic mean of the root length of the treated sample and $\bar{L}r_c$ is the arithmetic mean of the root length of the control:

$$\%RRG = \frac{\bar{L}r_s}{\bar{L}r_c} \times 100\% \quad (2)$$

After determining %RSG and %RRG, the GI was determined by Eq. 3:

$$\mathrm{GI} = \frac{\%RSG*\%RRG}{100} \quad (3)$$

Subsequently, the phytotoxicity level of the samples was rated according to Belo (2011) (Table 1).

Quantification of S-nitrosothiol and nitrite levels in lettuce radicles

Lettuce radicles were extracted by maceration with an aqueous solution of NEM (5 mmol L^{-1}) using a sonicator (45 kHz) for 10 min. The final homogenate was centrifuged (98,784×*g*) for 10 min, and 20 μL of supernatant was used for the amperometry quantification of RSNO and NO_2^- (Oliveira et al. 2016; Silveira et al. 2019). Measurements were carried out with the WPI TBR4100/1025 amperometer (World Precision Instruments Inc., Sarasota FL, USA) and a nitric oxide specific ISO-NOP sensor (2 mm). For quantification of RSNO, a solution of $CuCl_2$ (100 mmol L^{-1}) was used, and for NO_2^-, a solution of KI (100 mmol L^{-1}) in H_2SO_4 (100 mmol L^{-1}) was used. Data was compared to a standard curve obtained with S-nitrosoglutathione (GSNO) (for RSNO quantification) and $NaNO_2$ (for NO_2^- quantification) and normalized by radicle fresh weight.

Total antioxidant activity

The antioxidant activity (AA) of radicles was measured using DPPH radical photometric assay (Mishra et al. 2012). A volume of 300 μL of DPPH ethanolic solution (0.1 mmol L^{-1}) was added to 300 μL of radicle sample homogenized with 0.5 mL of PBS. The control group was prepared with DPPH and PBS. The final mixtures were kept at room temperature, protected from light for 30 min, and placed into quartz cuvettes. The absorbance intensities at 517 nm were recorded for all samples by using a UV-vis spectrophotometer (Agilent, model 8453, Palo Alto, CA, USA). The percentage of AA was calculated through Eq. 4, wherein Abs sample is the absorbance of the sample in ethanolic DPPH and Abs control is the absorbance of ethanolic DPPH and PBS. The assay was performed in triplicate and normalized by radicle weight.

$$\%AA = 100 - \frac{(\text{Abs sample}) \times 100}{\text{Abs control}} \quad (4)$$

Antioxidant enzyme activity

Lettuce radicles were ground to a powder with liquid nitrogen and then extracted with a medium composed of EDTA (1 mmol L^{-1}) potassium phosphate buffer (0.1 mol L^{-1}, pH 7.5) and PVP (2%, w/v). The extract

Table 1 Classification of phytotoxic levels

Germination Index (%)	Phytotoxic level
> 100	The material enhances germination and root growth of seeds
80–100	Non-phytotoxic, mature compound
60–80	Low
30–60	Moderate
< 30	High

was centrifuged at 15.645×*g*, at 4 °C for 20 min, and the supernatant fraction was used for the determination of enzymatic activities, as described below. The assay was performed in triplicate, and the activities were normalized by radicle weight and reaction duration.

Ascorbate peroxidase

An aliquot of the extract (50 μL) was mixed with 2.9 mL of EDTA (0.1 mmol L^{-1}), ascorbic acid (0.5 mmol L^{-1}), and potassium phosphate buffer (50 mmol L^{-1}, pH 7.0) (Nakano and Asada 1981). The absorbance at 290 nm was measured 2 min after the addition of 50 μL of H_2O_2 (30 mmol L^{-1}). The blank was acquired using the same solution without ascorbic acid. The enzymatic activity was calculated using Beer's law (ε = 2.8 L $mmol^{-1}$ cm^{-1}) (Shams et al. 2019).

Catalase

An aliquot of the extract (50 μL) was mixed with 0.95 mL of EDTA (0.1 mmol L^{-1}), H_2O_2 (12.5 mmol L^{-1}), and potassium phosphate buffer (50 mmol L^{-1}, pH 7.0). The absorbance change was measured for 3 min at 240 nm. The blank was acquired using the same solution without H_2O_2. The enzymatic activity was calculated using the Beer's law (ε = 36 L $mmol^{-1}$ cm^{-1}) (Anderson et al. 1995).

Peroxidases

An aliquot of the extract (20 μL) was mixed with 3.58 mL of pyrogallol (20 mmol L^{-1}), H_2O_2 (20 mmol L^{-1}), and potassium phosphate buffer (20 mmol L^{-1}, pH 6.8). After 1 min, concentrated H_2SO_4 (200 μL) was added to stop the reaction, and the absorbance was measured at 420 nm. The enzymatic activity was calculated using Beer's law (ε = 2.47 L $mmol^{-1}$ cm^{-1}) (Anderson et al. 1995).

Superoxide dismutase

The superoxide dismutase (SOD) activity is based on the assessment of inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Anderson et al. 1995). For that, an aliquot of the extract (40 μL) was mixed with 1.96 mL of EDTA (0.1 mmol L^{-1}), methionine (13 mmol L^{-1}), riboflavin (2 μmol L^{-1}), nitroblue tetrazolium chloride (75 μmol L^{-1}), and potassium phosphate buffer (50 mmol L^{-1}, pH 7.8) (Liu et al. 2014a). The reaction mixture was exposed with 300 μmol m^{-2} s^{-1} of photosynthetically active radiance, using a 60-W fluorescent lamp. After 10 min, an absorbance at 560 nm was measured. The reaction mixture containing distilled water instead of plant extract was also irradiated and used as control. The reaction mixture with plant extract and kept in the dark was used as blank. One unit of SOD was considered as the amount of the enzyme that inhibited NBT reduction by 50% (Liu et al. 2014a).

Statistics

A linear multi-regression was applied by considering the CuO NPs concentration (x) and enzymatic activities (y as APX, CAT, POD, SOD, NO_2^-, RSNO, AA%). A linear model was tested using the least squares method and the interpretation of beta coefficient ($p < 0.05$). A general model, where the variable response was equal to the model function plus the random error, was tested by residue analysis and one-way ANOVA ($p < 0.05$). The software Statistica 8.0 (Tulsa, USA) was used.

Results and discussion

CuO nanoparticle characterization

In this study, CuO NPs were biosynthesized using green tea extract, which acts as a reducing and capping agent. DLS measurements revealed a hydrodynamic size of CuO NPs: 70.1 ± 5.5 nm, PDI of 0.26 ± 0.01, and a zeta potential of 25.7 ± 2.1 mV (see Supplementary material 1). These results suggest the formation of NPs with low polydispersity. The negative value of zeta potential is assigned to the presence of negatively charged polyphenols (e.g., catechin) on the nanoparticle surface (Rolim et al. 2019a and 2019b). In addition, the magnitude of zeta potential indicates the thermal stability of the colloidal nanoparticle suspension due to sufficient electrostatic force between each nanoparticle. Similar results were reported by Yu et al. (2018) for CuO NPs (hydrodynamic size of 50 nm, zeta potential of 30 mV) obtained in a green synthesis protocol by using *Arbutus unedo* leaf extract. Similarly, Dey et al. (2019) reported the synthesis of CuO NPs using *Azadirachta indica* leaves, obtaining a hydrodynamic size of 54 nm, a zeta potential of 28.1 mV, and a PDI of 0.25.

A representative image of CuO NPs, at solid state, acquired by TEM, showed the formation of spherical shape CuO NPs with an average size of 6.6 ± 0.2 nm (Fig. 1a, b). The NPs are well dispersed and have a small size at solid state. These results were similar to the data reported by Naika et al. (2015) for CuO NPs synthesized using *Gloriosa superba* L. extract, in which the NPs had a spherical shape and size in the range of 5–10 nm.

The elemental state of green tea-synthesized CuO NPs was evaluated by XPS analysis (Fig. 2). The survey spectrum shows the presence of carbon, oxygen, and copper (Fig. 2a), due to the formation of pure CuO NPs coated by green tea compounds. The high-resolution core level spectrum of Cu 2p at 950.86 eV (Cu 2p1/2) and 931.0 eV (Cu 2p3/2) can be observed in Fig. 2b. The band of Cu 2p1/2 can be deconvoluted in the other two bands, located at 953.0 eV and 950.0 eV and 933.0 eV and 931.0 eV for Cu 2p3/2. These results are close to the data reported by Nagajyothi et al. (2017). The non-appearance of satellite peaks of open 3d9 shell indicates the absence of the Cu^{+} state (Nagajyothi et al. 2017). Furthermore, the gap between the Cu 2p3/2 and Cu 2p1/2 was 19.8 eV, which is similar to the standard value of 20.0 eV for Cu 2p of CuO (Bhattacharya et al. 2019). Figure 2 c presents the high-resolution C1s spectrum. The peak located at 531.0 eV can be attributed to carbon-based compounds coated on the NP surface, as previously reported for green synthesized CuO NPs (Potbhare et al. 2019). The high-resolution spectrum of O1s (Fig. 2d) shows a band located at 532.24 eV, which can be attributed to the presence of O^{2-} bonded with copper, due to the formation of CuO NPs (Potbhare et al. 2019).

Seed germination and radicle elongation

After 5 days of CuO NPs exposure, the seed germination index (Table 2) and radicle elongation (Fig. 3a) were measured. No significant alterations ($p < 0.05$) in radicle elongation were observed between 0.2 and 40 μg mL^{-1} NP concentrations. Interestingly, with 20 μg mL^{-1}, enhanced seed germination and radicle growth were enhanced compared to control. For seed germination, no toxicity was demonstrated for concentrations ranged between 0.2 and 40 μg mL^{-1}. However, from 80 μg mL^{-1}, the phytotoxicity of CuO NPs considerably increased (from 65% of GI%), up to 150 μg mL^{-1} with GI of 32% and 300 μg mL^{-1} with a GI of 24% which is classified as strongly phytotoxic.

Some reports presented similar results for other edible plants. The application of Cu NPs (< 100 nm in diameter) at 500 mg L^{-1} in pumpkin (*Curcubita* L.) seed bioassays resulted in 60% inhibition of root growth after a 14-day-incubation (Musante and White 2012). On the other hand, 100 mg L^{-1} did not result in inhibitory effects (Musante and White 2012). Nevertheless, concentrations of 0.1, 1, and 10 mg L^{-1} of CuO NPs caused more toxic effects in root elongation of rice seeds (*Oryza sativa* japonica "Koshihikari") in comparison with 50 and 100 mg L^{-1} concentrations after an 18-day incubation (Liu et al. 2018). This can be explained by the possible synthesis of metabolites by plants able to

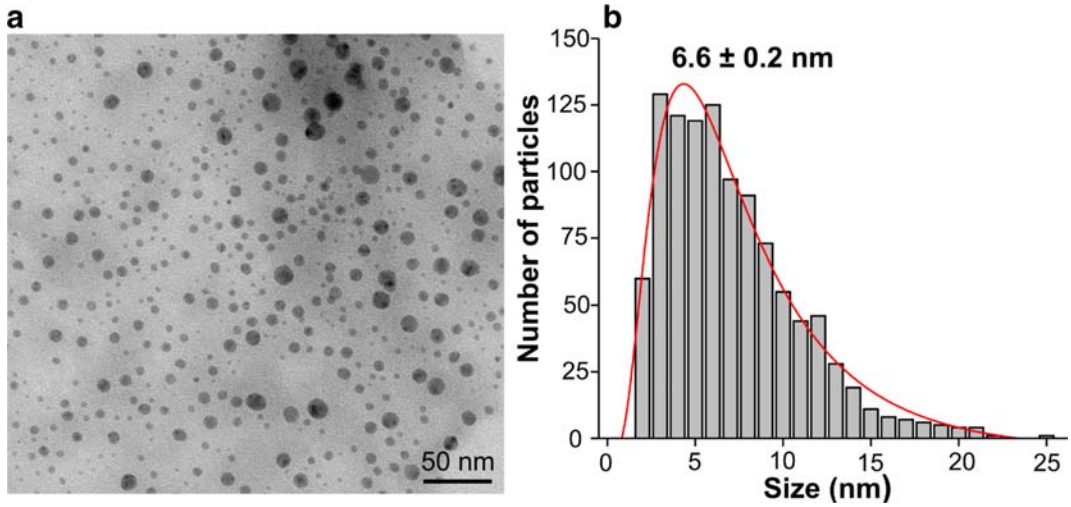

Fig. 1 **a** Representative TEM image and **b** size distribution of green tea-synthesized CuO NPs

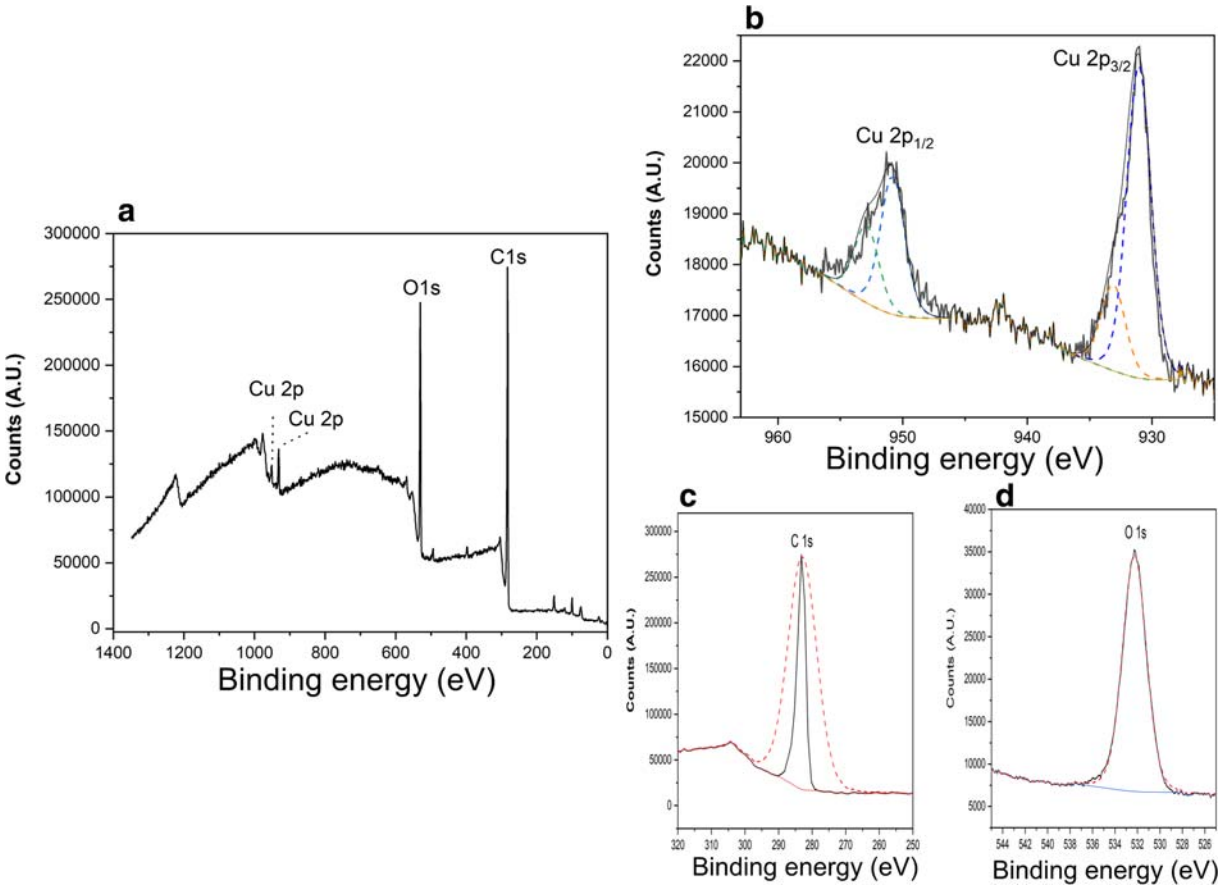

Fig. 2 **a** Survey spectrum of CuO NPs. High-resolution spectra of **b** Cu2p region, **c** C1s region, and **d** O1s region, assayed by XPS analysis

minimize the toxicity of metallic NPs, decreasing the total toxic effect caused on the seed (Štefanić et al. 2018). The diameter of NPs is also an important factor triggering phytotoxic effects on seed germination and radicle growth in these bioassays. For instance, the application of 50 mg kg^{-1} of 280 nm diameter Cu-based NPs in cilantro (*Coriandrum sativum*) resulted in 60% inhibition of root growth against only 40% for a 2590 nm diameter for 30 days of incubation (Zuverza-Mena et al. 2015).

Considering lettuce seeds, Liu et al. (2016) reported prominent inhibition of lettuce germination in a 5-day

Table 2 Germination Index values (%) for lettuce seeds treated with CuO NPs at 0.2, 2, 20, 40, 80, 150, and 300 μg mL^{-1}

CuO NP (mg L^{-1})	Average GI (%)	Rating according to Belo (2011)
0	100	Control sample
0.2	94.86 ± 11.43a	Non-phytotoxic
2	93.86 ± 15.91a	Non-phytotoxic
20	101.45 ± 11.62b	Enhances germination and radicle growth
40	89.28 ± 19.30a	Non-phytotoxic
80	65.49 ± 9.83c	Moderately phytotoxic
150	32.18 ± 16.58d	Phytotoxic
300	24.84 ± 16.48e	Strongly phytotoxic

Lowercase letters indicate statistical difference by Tukey test ($p < 0.05$)

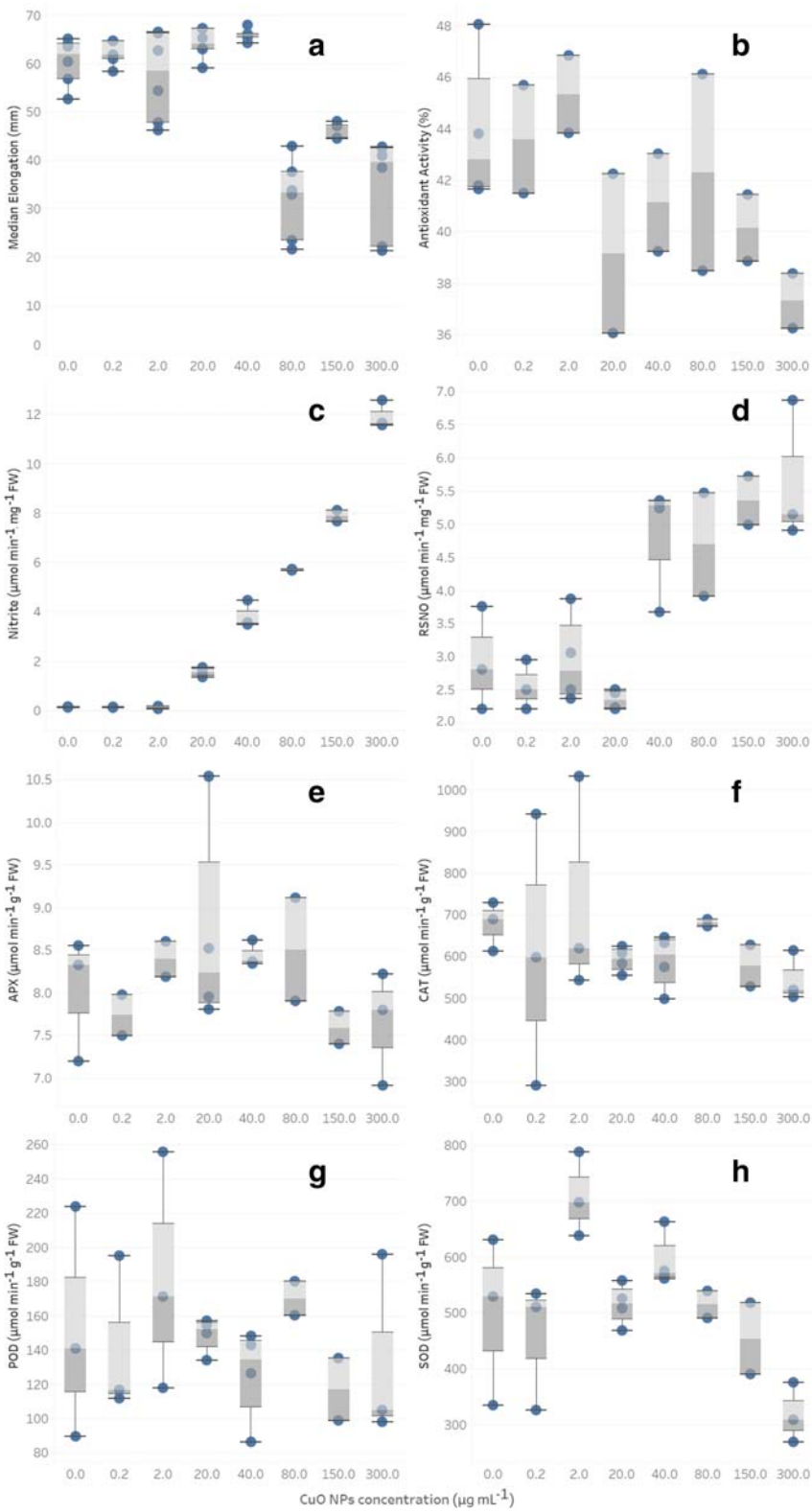

Fig. 3 Dose effects of green tea-synthesized CuO NPs on **a** radicle elongation, **b** antioxidant activity, **c** nitrite concentration, **d** RSNO concentration, **e** APX activity, **f** CAT activity, **g** POD activity, and **h** SOD activity

petri dish test conducted with seeds exposed to CuO NPs (10 ± 8 nm) at very low concentrations (0.02 to 8 μg mL^{-1}). Hong et al. (2015) evaluated three Cu-based NPs and other Cu compounds in hydroponically grown lettuce and alfalfa (*Medicago sativa*) at concentrations of 0, 5, 10, and 20 mg L^{-1}, and their results suggest that Cu-based compounds not only reduced the size of the plants but also altered the nutrient content and enzyme activity in both plant species. Trujillo-Reyes et al. (2014) demonstrated that the application of Cu/CuO NPs in hydroponically-grown lettuce seedlings (18-day-old) treated for 15 days at concentrations of 10 and 20 mg L^{-1} affected the plant growth, water content, and concentration of several nutrients. At 10 mg L^{-1}, the nano Cu material increased CAT and decreased APX in roots. Comparing our findings with those of previous reports regarding bioassay studies conducted with lettuce seeds exposed to non-biogenic Cu-based NPs, it is possible to observe a notable difference in the inhibition of seed germination and radicle growth, suggesting a higher tolerance of lettuce seeds to green tea-synthesized CuO NPs.

Reactive oxygen species and nitric oxide response to biogenic CuO nanoparticle application

In this study, the relationships between the independent variable (exposure to different concentrations of CuO NPs) and dependent variables (total antioxidant activity (%AA), SOD, POD, CAT, APX, NO_2^-, and RSNO) were established. The selected CuO NPs concentrations (0, 0.2, 2, 4, 40, 150, and 300 μg mL^{-1}) addressed the range issue on the regression analysis, where the range should be enough to achieve a desired effect and be narrow enough to obtain a simple model function (Draper and Smith 1998).

Figure 3 b shows a decreasing trend of antioxidant activity (− 0.57) (Table 3), with an increase of CuO NP concentration. An inverse and weak correlation (− 0.59) (Table 3) was also observed between %AA and NO_2^-, which indicates both variables were affected by CuO NPs exposition.

NO_2^- and RSNO levels (Fig. 3c, d) suggested a linear response with CuO NPs concentration (R^2 = 0.97 and 0.68, respectively) (Table 3). One-way ANOVA was used to evaluate the variances between CuO NP concentrations. Significant statistical differences were observed between values from 0 up to 40 and 80 up to 300 μg mL^{-1} for NO_2^- and RSNO. No significant difference was observed for the other variables. However, this could be related to the high variance observed among low concentration values (0 to 2.0 μg mL^{-1}), which were observed for CAT, POD, and SOD.

The linear response was confirmed, by the beta coefficient (see Supplementary material 2), as being associated only with the NO_2^- values (Fig. 4a). The excess of Cu^{2+} may have a negative impact on the nitrite reductase (NR) enzyme. NR is a cytosolic enzyme responsible for catalyzing the one-electron reduction of NO_2^- to NO and is dependent on NAD(P)H (Besson-Bard et al. 2008). The excess of Cu^{2+} may decrease the NR gene expression (Hippler et al. 2018). The CuO NPs can generate copper ions that will have this negative impact on NR activity, accumulating NO_2^- levels. At the same time, CuO NPs may increase NO formation.

The RSNO levels also increased as the CuO NPs concentration administered increased. The highest CuO NPs concentration administered (300 μg mL^{-1}) showed ca. 6 μmol of RSNO per mg of radicle (Fig. 3d). This increase in the endogenous level of RSNO and NO_2^- may indicate a toxicity upon seed exposure to a high concentration of CuO NPs, which also reduced radicle growth at 50, 150, and 300 μg mL^{-1} of CuO NPs, as assessed by a germination test (Fig. 3a). Anderson et al. (2018) showed similar results with wheat seedling root treated with CuO NPs, and the NO quantification was measured by confocal microscopy with 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM DA).

The effects of CuO NPs on ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities are shown in Fig. 3e–h, respectively. As expected, a close association was observed among the responses of APX, CAT, POD, and SOD, with correlation coefficients around 0.7 (Fig. 4c). However, only SOD activity decreased with an increase of CuO NPs concentration (Fig. 3h); thus, a low inverse correlation was observed (− 0.55) (Table 3). An interesting finding was that SOD activity and nitrite levels were inversely correlated (− 0.50) (Table 3).

Under oxidative stress, the plant antioxidant system is activated, and SOD, which converts O_2^- to O_2 and H_2O_2, is the first enzyme to act. NO regulates antioxidant enzymes at the level of activity and gene expression, which can cause either enhancement or

Table 3 Observed correlations between variables of Lettuce seedlings (*Lactuca sativa* L.) treated with concentrations of CuO NPs (0.2, 2, 20, 40, 80, 150, and 300 μg mL^{-1}). The variables correlated were CuO NPs concentration, catalase (CAT), superoxide dismutase (SOD), and peroxidases (POD), ascorbate peroxidase (APX), total antioxidant activity (%AA), nitrite (NO_2^-), and S-nitrosothiol (RSNO)

	Conc.	RSNO	APX	CAT	POD	SOD	NO_2^-	%AA
Conc.	1.00							
RSNO	*0.68*	1.00						
APX	− 0.43	− 0.10	1.00					
CAT	− 0.04	0.04	0.28	1.00				
POD	− 0.34	− 0.24	0.46	0.31	1.00			
SOD	*− 0.55*	− 0.20	*0.53*	0.33	*0.59*	1.00		
NO_2^-	*0.97*	*0.78*	− 0.34	0.01	− 0.30	*− 0.50*	1.00	
%AA	*− 0.57*	− 0.27	0.41	− 0.01	0.00	0.22	*− 0.59*	1.00

Results with $|r| \geq 0.5$ ($p < 0.05$) are highlighted in italics

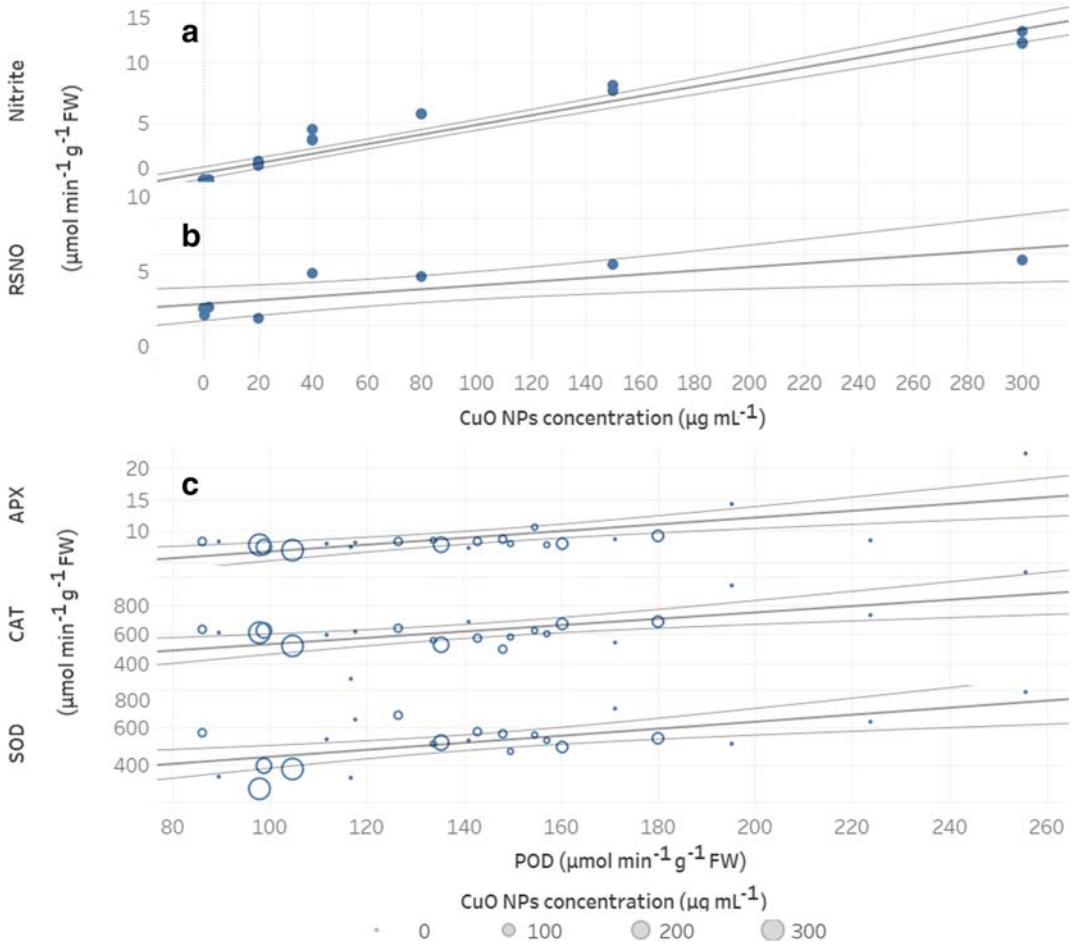

Fig. 4 Linear regression of **a** nitrite levels versus CuO NP concentration. **b** RSNO levels versus CuO NP concentration. **c** Linear regressions between APX activity, CAT activity, POD activity, and SOD activity

reduction of the cellular redox status (Groß et al. 2013). It has been demonstrated that, although S-nitrosation is related to SOD stimulation (Sehrawat and Deswal 2014), some SOD isoenzymes are inhibited by peroxynitrite-mediated tyrosine nitration (Holzmeister et al. 2015). The findings presented here might suggest that lettuce seedlings exposed to green tea-synthesized CuO NPs at higher concentrations (> 20 μg mL^{-1}) trigger RNS production, as evidenced by NO_2^- and RSNO increase, and, finally, that NO can decrease the SOD activity and the antioxidant metabolism.

Conclusion

This study showed the synthesis and characterization of ultra-small CuO NPs using a commercial green tea extract. Characterization by several techniques indicated the formation of CuO NPs capped with green tea polyphenols, at the nanoscale. This work provides the first evidence, to our knowledge, that green tea-synthesized CuO NPs at concentrations between 0.2 and 20 μg mL^{-1} are non-phytotoxic and might enhance lettuce radicle growth. However, at higher concentrations (40 to 300 μg mL^{-1}), these NPs decreased radicle growth in lettuce. It is clear that the activity of antioxidant enzymes, such as superoxide dismutase, and the levels of RNS, such as nitrite and RSNO, were affected by NP exposition. This novel finding indicates that the sharp increase of nitrite levels with the rising of NP concentration is related to the decrease of SOD and total antioxidant activity. Copper-NP uptake, distribution, and effects on molecular and genetic levels of lettuce plants are beyond the scope of this study but are strongly recommended to be taken into account in future studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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