

Exploration the homeostasis of signaling molecules in monocotyledonous crops with different CuO nanoparticle tolerance

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ABSTRACT

Copper is an essential microelement that is indispensable for plant growth and development. The use of copper oxide nanoparticles (CuO NPs) in industry and agriculture has also increased because of their beneficial properties. However, excess amounts of CuO NPs may negatively affect the growth of monocotyledonous plant species, primarily through the generation of reactive oxygen species, which results in oxidative stress. Despite their increasingly widespread use, little is known regarding the signaling processes responsible for the effects of CuO NPs on the growth of monocotyledonous crops, or their impact on the homeostasis of reactive nitrogen species, hydrogen sulfide, and protein tyrosine nitration.

In this study, the concentration of CuO NP that inhibits 50% of root growth was determined using sorghum, wheat, rye, and triticale as model plant species, and the NP-induced stress response and the balance of reactive molecules were assessed. Based on the effective concentration of CuO NP, wheat, rye, and triticale were more tolerant compared with sorghum, and entirely different response mechanisms in the homeostasis of reactive oxygen, nitrogen and sulfur species were observed. For the sensitive sorghum roots, the amount of reactive molecules was not significantly altered, whereas a significant increase in protein tyrosine nitration indicated a severely stressful state caused by CuO NPs. In contrast, the amount of reactive molecules increased significantly in the roots of the relatively tolerant species, and while the appearance of lipid peroxidation indicated oxidative stress, different changes in protein tyrosine nitration was associated with tolerance. The significant CuO NP-induced rise of endogenous H₂S content in the root tips may be partly responsible for the relative tolerance of wheat, rye, and triticale compared with sorghum. CuO NP stress induced distinct modifications in the root tip cell walls of the examined species, where lignification was observed in the relatively sensitive sorghum, while in the tolerant species only callose deposition was detected. Overall, our results demonstrate that while monocotyledonous species with different CuO NP sensitivities may exhibit similar growth responses, the underlying changes in the dynamics of reactive molecules influence their tolerance.

1. Introduction

Copper, a transition metal with an atomic number of 29, has been a key metal throughout the history of mankind because of its ease of processing. Copper is a chemically and biologically active metal because its ions can readily accept and donate electrons, thus exhibiting a high redox potential and generating reactive oxygen species (ROS) (Foley et al., 2002). Copper is an essential micronutrient for plants, but is toxic at high concentrations. Copper is used by plants during photosynthesis, mitochondrial respiration, and the oxidative stress response (Marschner

2011). Because of its oxidation state, copper oxide is more toxic compared with elemental copper (Rastogi et al., 2017).

Copper oxide nanoparticles are used in many fields, such as the replacement of copper in fertilizers, catalysts, superconductors, thermoelectric sensors, glasses, ceramics, antibacterial treatments, photo-sensitive electrical devices, magnetic data storage, and semiconductors (Ochoa et al., 2018). Because of their wide range of applications, they have a very large number of vectors for entry into the environment and it is important to address the impact. One important pathway of entry is the agro-technical use of wastewater, in which nanoparticles may come

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into direct contact with agricultural crops (Brar et al., 2010).

Copper oxide nanoparticles can affect plants in two ways: they can release copper or they can cause particle stress following entry into the plant (Ruttkey-Nedecky et al., 2017). The primary mechanism of phytotoxicity is the generation of ROS and the oxidation of lipids, whereas the second mechanism involves the release of copper ions from particles (Du et al., 2021). In addition, there is evidence that copper oxide nanoparticles are capable of damaging DNA (Atha et al., 2012). For nanoparticles to enter plants and exert their effects, they must first pass through the cell wall, a structure composed primarily of cellulose which allows small particles to pass through as the permeability limit is below 5–20 nanometers (Dietz and Herth 2011). There is evidence that some nanoparticles can form pores in the cell wall, allowing larger particles to enter (Kurczyńska et al., 2021). The nanoparticles may then undergo endocytosis and are transported through the symplast to other tissues in the plant (Ma et al., 2010). It is not yet clear whether copper oxide nanoparticles are more toxic (i.e., they inhibit plant growth at the same concentration) compared with larger-sized copper oxide particles (Kumar et al., 2021; Roy et al., 2022). Copper ions released from the particles are able to bind to the thiol groups of proteins, causing the proteins to undergo conformational changes (Nekrasova and Maleva, 2007). Another mechanism of toxicity occurs through the Fenton reaction, in which copper ions convert hydrogen peroxide into hydroxyl radicals, which in turn, damage the surrounding macromolecules (Chung et al., 2019).

Oxidative stress is attributed to an imbalance between the production of ROS, such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) (Jain et al., 2010; Morina et al., 2010; Gill and Tuteja, 2010) and the protective effects of antioxidants (Corpas and Barroso, 2013). To ensure the survival of plants, ROS levels are tightly regulated by a complex mechanism (Apel and Hirt, 2004) that includes several enzymatic or non-enzymatic antioxidants. Overproduction of ROS negatively affects plant cells at several levels by damaging genetic material, proteins, and lipids. ROS can directly modify proteins by oxidation of amino acid side chains or by reactions with aldehyde products of lipid peroxidation or glycosylation, which results in carbonyl groups in the protein molecule (Madian and Regnier, 2010).

In addition to ROS, reactive nitrogen species (RNS) may also be formed as a result of a number of environmental stresses (Arasimowicz and Floryszak-Wieczorek, 2007). RNS refers to nitric oxide (NO) and related molecules including peroxyxynitrite ($ONOO^-$) and S-nitrosoglutathione (GSNO) (Wang et al., 2013). Nitrosative stress, as an equivalent to oxidative stress, results from the accumulation of the above-mentioned molecules in cells, which can be caused by a variety of environmental factors (Corpas et al., 2007, 2011). The metabolism of ROS and RNS is linked at numerous points and the idea of nitro-oxidative stress has recently become an area of interest in plant biology (Corpas et al., 2013). A representative example of the ROS-RNS interaction is the reaction of O_2^- and NO, which produces $ONOO^-$ and is responsible for the covalent alteration of particular tyrosine residues in proteins, resulting in protein tyrosine nitration. The addition of a nitro group to one of the ortho carbon atoms of the aromatic ring of tyrosine (Gow et al., 2004) modifies the ability of tyrosine to maintain the correct conformation of a protein. The most common result of this is loss of protein function, but infrequently, a gain-of-function or modification without effect has been reported (Corpas et al., 2013; Greenacre and Ischiropoulos, 2001; Radi, 2004). Furthermore, tyrosine nitration can interrupt signaling pathways by inhibiting tyrosine phosphorylation.

In addition to NO, hydrogen sulfide (H_2S) also plays an important role in plant signaling pathways, seed germination, and subsequent fruit ripening; however, it is also involved in the abiotic/biotic stress response. Similar to NO, H_2S can form reactive free radicals and is an important member of the reactive sulfur species (RSS) family of molecules. As with NO, only the adverse effects of H_2S were previously known, but recent studies have focused on its beneficial effects (Corpas et al., 2019; Corpas and Palma 2020). It has also been found that the

biochemical regulation of NO and H_2S is clearly associated with the metabolism of ROS, suggesting a biochemical link between molecular families (Rodríguez-Ruiz et al., 2017).

The effects of copper oxide nanoparticles have been investigated in a relatively few number of plant species. In most cases, monocots did not respond or responded negatively to copper oxide nanoparticle treatment. Among those, wheat (*Triticum aestivum*) subjected to copper oxide nanoparticle treatment exhibited a positive effect on early plant development at low concentrations, but was inhibitory at higher concentrations (Dimpka et al., 2012; Adams et al., 2017; Guan et al., 2020; Badawy et al., 2021; Ibrahim et al., 2022). Other species that were positively influenced by copper oxide nanoparticles were predominantly dicotyledonous.

As studies comparing CuO nanoparticle stress-induced changes in the reactive signaling balance in the roots of monocotyledonous crops are scarce, the aim of the present study was to induce 50% root growth inhibition by copper oxide nanoparticles on sorghum (*Sorghum bicolor* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), and triticale (*x Triticosecale*) as model plants, and determine the changes in the dynamics of reactive signaling molecules behind the growth response. In addition to its known effect on ROS, it has been hypothesized that CuO may disrupt RNS and RSS homeostasis, leading to changes in the nitroproteome of the plant roots.

Because of the rapid development of nanotechnology, metal oxide nanoparticles, including CuO NPs, have become important to agriculture, but their impact is not yet fully understood. Thus, it is important to evaluate these substances as soon as possible in case they have deleterious effects on human health.

2. Materials and methods

2.1. Synthesis and characterization of copper nanoparticles (CuO NPs)

CuO nanoparticles were synthesized by a modified precipitation method based on the work of Phiwang et al. (2013) and Molnár et al. (2020b). First, copper nitrate [$Cu(NO_3)_2 \times 3H_2O$] precursor was dissolved in 100 ml deionized water to form 0.2 M concentration. Next, 25% ammonia solution was slowly added dropwise while vigorous stirring until the pH reached 2. The color of the solution turned from blue to black immediately and resulted in the formation of a black precipitate. This black precipitate was repeatedly washed with deionized water and absolute ethanol several times until the pH reached 7. Subsequently, the washed precipitate was dried at 60 °C overnight to remove any remaining solvent, then calcined at 450 °C for 2 h using a tube furnace in air. Finally, the resulting powder was ground and stored at room temperature until further use. All chemicals used for the synthesis of CuNPs were analytic reagent grade. Copper nitrate [$Cu(NO_3)_2 \times 3H_2O$], ammonia solution, and ethanol were purchased from Merck (Darmstadt, Germany). Deionized water was used throughout the experiments.

The morphological characteristics of the CuO NPs were analyzed by transmission electron microscopy (TEM) using an FEI Tecnai G2 20× microscope (FEI Corporate Headquarters, Hillsboro, OR, USA) with an acceleration voltage of 200 kV. The crystal structure and phase of the nanoparticles were verified by X-ray powder diffraction (XRD). The scans were performed using a Rigaku MiniFlex II powder diffractometer (Rigaku Corporation, Tokyo, Japan) with Cu $K\alpha$ radiation. A scanning rate of 2° min⁻¹ in the 10°–70° 2θ range was used. The absorbance spectra of the nanoparticles were recorded within a range of 200–600 nm using an Ocean Optics 355 DH-2000-BAL UV–Vis spectrophotometer (Halma PLC, Largo, FL, USA) with a 10-mm path length quartz cuvette.

2.2. Plant material and growing conditions

Sorghum (*Sorghum bicolor*, GK Emese), wheat (*Triticum aestivum*, GK Békés), rye (*Secale cereale*, Wibro), and triticale (*x Triticosecale*, GK

Maros) seeds were obtained from the Cereal Research Non-profit Ltd, Szeged, Hungary. During the experiments, an *in vitro* system was used, in which the surface sterilized (1 min 70 v/v% ethanol, 5 min 5% sodium hypochlorite) seeds (ten seeds per dish) were placed in Petri dishes (diameter: 9 cm) lined with two layers of filter paper (1602/N grade). The filter paper was moistened with 5 mL distilled water (control) or with the same amount of aqueous solution of CuO NPs (dispersed using ultrasound sonicator, pH set to 5.7–5.8). The closed Petri dishes were maintained under controlled conditions in a greenhouse: 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (white LED 5700 K) with far red illumination (PSI, Drásov, Czech Republic), 12/12-hour light/day cycle, day/night temperature of 24 °C/22 °C, and 55%–60% relative humidity for 5 days (Molnár et al., 2020a,b).

Preliminary experiments were performed to determine CuO NP concentrations that cause 50% growth inhibition (in terms of root length) in each species. According to these results, 50 mg/L CuO NP was used for sorghum seeds and 150 mg/L CuO NP was used for wheat, rye, and triticale seeds (Suppl. fig. 1).

2.3. Root growth parameters

At the end of the growing period, the primary root length (PR length; mm) of sorghum was measured and the visible lateral roots were counted (LR; pieces/primary root). For wheat, rye, and triticale, the number of developed fibrous roots were counted (root number, RN; pieces/seedling) and their length were measured (FR length; mm). The fresh weight of the root system was also measured (RFW; mg).

2.4. Detection of reactive signal molecules

Fluorescent microscopic dyes were used to detect the presence and monitor changes in the levels of reactive signal molecules in the root tips. For superoxide, 10 μM dihydroethidium (DHE) in 10 mM Tris/HCl, pH 7.4, was used (Petó et al., 2013). 50 μM Ampliflu™ (10-acetyl-3, 7-dihydroxyphenoxazine, ADHP, or Amplex Red in 50 mM sodium-phosphate buffer, pH 7.5) was used to detect fluorescence associated with hydrogen peroxide (Lehotai et al., 2012). Nitric oxide-dependent fluorescence was measured using 10 μM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA; prepared in 10 mM Tris/HCl, pH 7.4) (Kolbert et al., 2012). For the detection of fluorescence associated with the presence of peroxyxynitrite, 10 mM 30-(p-aminophenyl) fluorescein (APF; prepared in 10 mM Tris/HCl, pH 7.4) was used (Chaki et al., 2009). Hydrogen sulfide linked fluorescence was detected using Washington State Probe-1 (WSP-1; 15 μM , prepared in 20 mM Hepes-NaOH, pH 7.5) (Li et al., 2014).

2.5. Microscopic determination of glutathione, quercetin callose, lignin, and lipid peroxidation in root tissues and the viability of the root apical meristem

Glutathione in the cells of the root tip was detected with monobromobimane (MBB; 100 μM , prepared in distilled water) (Meyer et al., 2001).

Diphenylboric acid 2-amino-ethylester (DPBA; 0.25 w/v%, prepared in distilled water with 0.005 v/v% Triton X-100) was used to visualize quercetin in the root tips (Sanz et al., 2014; Molnár et al., 2020b). DPBA emits fluorescence with distinct spectral properties in complex with kaempferol and quercetin, where in our experimental system the measured golden yellow fluorescence related to quercetin.

Callose content in the cell walls of the root tips was detected by aniline blue staining (0.1%, w/v in 1 M glycine) (Feigl et al., 2015). Lignin in the root cell walls was detected with phloroglucinol-HCl (1 w/v% phloroglucinol prepared in 6 N HCl) (Rogers et al., 2005). Schiff reagent was used to visualize lipid peroxidation in the root tips (20 min incubation with the reagent followed by a 20 min incubation in 0.5 w/v % $\text{K}_2\text{S}_2\text{O}_5$ solution in 0.05 M HCl) (Arasimowicz-Jelonek et al., 2009).

Viability (proportional to intracellular esterase activity) of the root apical meristem was determined with fluorescein diacetate [FDA, 10 μM , prepared in 10 mM MES (4-morpholineethanesulfonic acid)/50 mM KCl buffer, pH 6.15] staining (Lehotai et al., 2011).

2.6. Acquisition and processing of microscopic images

Root tip sections treated with various fluorescent probes were examined under a Zeiss Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany). Filter set 9 (exc.: 450–490 nm, em.: 515– ∞ nm) was used for DHE, DPBA, and WSP-1, filter set 10 (exc.: 450–490, em.: 515–565 nm) was applied for APF, DAF-FM, and FDA, filter set 20HE (exc.: 546/12, em.: 607/80) was used for AmplexRed, and filter set 49 (exc.: 365 nm, em.: 445/50 nm) was used with aniline blue, MBB. With respect to imaging, the fluorescence intensity (pixel intensity, in correlation with the amount of the detected substance) was measured in the meristematic zone of the root tips using Axiovision Rel. 4.8 software within circles of 50 μm radii.

2.7. Western blot detection of nitrated proteins

Protein extracts were prepared from the seedling root tissues as described by Kolbert et al. (2018). Then, 15 μL of each extract was separated on 12% acrylamide gels (SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis) following the procedure described by Kolbert et al. (2018) to detect nitrated protein bands.

2.8. Statistical analysis

The results are expressed as the mean \pm standard error (s.e.). Multiple comparison analyses were done with SigmaStat 12 software using an analysis of variance (ANOVA; $P < 0.05$) and Duncan's test.

3. Results and discussion

3.1. Characterization of CuO NPs

The CuO nanoparticles were visualized by TEM, which indicated that they were ellipsoid and rod-like in shape. Minor polydispersity was observed and the particles formed loose aggregates (Fig. 1A). The average size of the particles was 48.2 ± 6.3 nm. XRD studies were performed to verify the crystalline structure and chemical composition of the synthesized nanoparticles (Fig. 1B). The characteristic peaks located at $2\theta = 32.53^\circ, 35.52^\circ, 38.87^\circ, 48.74^\circ, 61.53^\circ,$ and 68.24° were assigned to (110), (002), (200), (–202), (–113), and (220) plane orientation for the monoclinic structure of CuO (JCPDS 892,531; Suresh et al., 2016). No peaks associated with impurities were observed in the XRD pattern. The UV–Vis spectra (Fig. 1C) further confirmed the chemical composition of the sample, as an absorption maximum around 298 nm represented the characteristic value based on the literature (Jillani et al., 2018).

3.2. CuO NP-induced root morphological changes

The first goal that forms the basis of the work was to determine the CuO NP concentration that induces 50% root growth inhibition, in terms of root length. Sorghum was more sensitive compared with wheat, rye, and triticale, as 50 mg/L CuO NP treatment was able to induce the anticipated root growth response, whereas in the others, 150 mg/L of CuO NP was required for the same effect. Although longitudinal root growth was significantly inhibited, fibrous root numbers of wheat, rye, and triticale seedlings decreased only slightly and the lateral root formation of sorghum was not affected by CuO NP stress. Because of the inhibition of root elongation, the fresh weight of the root system was also significantly decreased by CuO NP stress in all the plant species examined (Table 1).

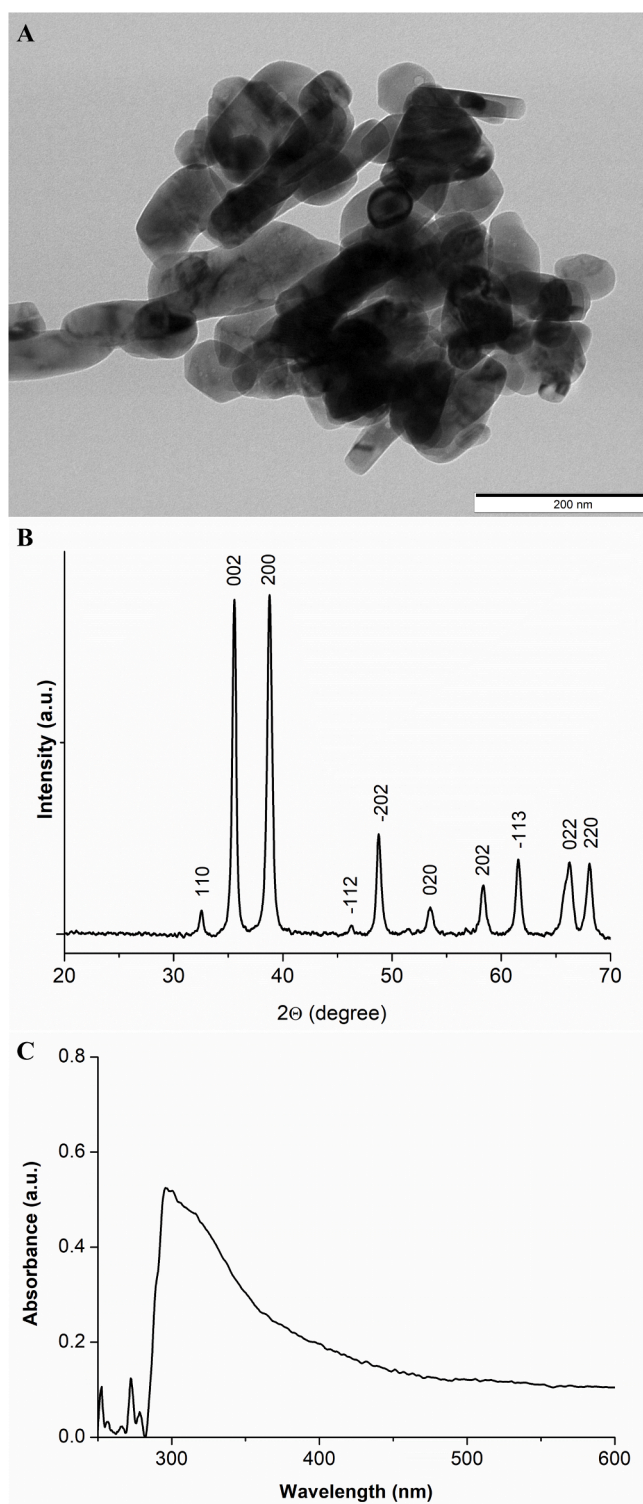


Fig. 1. Characterization of copper oxide nanoparticles. Transmission electron microscopic (TEM) image (A), and X-ray diffractogram (XRD) of the synthesized particles with highlighted characteristic Miller indices (B), UV-Vis spectrum (C). Bar=200 nm.

The metabolic activity of the root apical meristem detected by FDA staining may be used as a marker of root tip vitality or viability (Feigl et al., 2019). CuO NP stress significantly decreased apical meristem viability of the relatively sensitive sorghum. Among the relative tolerant species, growth-inhibiting CuO NP treatment significantly decreased the viability of wheat root tips, whereas in rye and triticale, only some minor

inhibition was detected (Table 1). While the root growth results and the root tip viability data may appear contradictory in the last two species, root elongation is regulated by a complex network of factors (e.g., metabolic, hormonal, etc.), not just the metabolic activity of the apical meristem cells (Satbhai et al., 2015).

3.3. Changes in the dynamics of reactive signaling molecules in the root tips

In addition to stress-induced root growth responses, a disturbance in the balance of reactive signal molecules can often be detected (e.g., water stress: Signorelli et al., 2013; heavy metals: Georgiadou et al., 2018; indium: Zhao et al., 2022). Thus, we examined the potential variances in this network between the different CuO NP sensitivity of the monocotyledonous species. CuO NP-induced changes in ROS, RNS, and RSS homeostasis was detected in the meristematic zone of the root tips. In general, whereas the trend of the alterations was mostly similar in all monocot species, a significant difference was observed in the extent of changes between the relatively tolerant wheat, rye, and triticale species and compared with the relatively sensitive sorghum.

The superoxide anion content of the root tips of sorghum was not affected by 50 mg/L CuO NP stress. In contrast, 150 mg/L (50% root growth-inhibiting) CuO NP treatment significantly increased superoxide anion levels in the meristematic zones of the relative tolerant species (Fig. 2A). Similarly, CuO NP stress significantly induced the accumulation of hydrogen peroxide in the root tips of wheat, rye, and triticale. In contrast to superoxide, the amount of hydrogen peroxide was slightly, but visibly increased, in the root tips of sorghum (Fig. 2B).

The appearance of pink coloration associated with lipid peroxidation correlated with high ROS levels in the root tips, because it was observed in the relatively tolerant species (Fig. 2D). CuO NP stress induced the accumulation of H_2O_2 , which is primarily responsible for the appearance of malondialdehyde (lipid peroxidation), a marker of oxidative stress (Del Rio et al., 2005). Glutathione, as a non-enzymatic antioxidant, is important for the detoxication of ROS, or the hydrogen peroxide in plant cells (Hasanuzzaman et al., 2017). A comparison of the amount of GSH and hydrogen peroxide in the root tips indicated that the relatively sensitive sorghum contained the most GSH under control circumstances, but was decreased significantly with increasing H_2O_2 during CuO NP stress (Fig. 2B and C). In the relative tolerant species, there was no significant difference in GSH content in the CuO NP-stressed root tips; however, both GSH content in the control roots and the degree of reduction were different in the three species (Fig. 2C).

In contrast to the stress caused by Cu, stress induced by CuO NPs was only assessed in a few monocot plants, and most of them were evaluated for possible growth responses, and not the underlying mechanism responsible. Similar to our results, a previous study showed that the root growth of wheat was inhibited by 500 mg/kg CuO NP and accompanied by increased lipid peroxidation as evidence of oxidative stress (Dimpka et al., 2012). Similarly, in rice, both 100 μ M and 250 mg/L CuO NP reduced root growth through oxidative damage (Rai et al., 2021; Yang et al., 2020). Recently, Roy et al. (2022) found that bulk CuO particles were more toxic to maize compared with CuO nanoparticles; however, CuO NP-induced growth inhibition was associated with significant ROS generation and lipid peroxidation.

Compared to the relationship between CuO NPs and ROS, less is known regarding the effect of CuO NPs on the homeostasis of RNS. The tendency of CuO NP-induced changes in RNS was similar to that for ROS. In the relatively sensitive sorghum root tips, CuO NP stress induced only slight NO accumulation; however, among the control roots, NO content was the highest of all species examined. On the other hand, in the relatively tolerant wheat, rye, and triticale, growth-inhibiting CuO NP stress induced significant NO accumulation (Fig. 2E). In contrast, during CuO NP-induced growth inhibition of rice roots, NO levels decreased significantly (Rai et al., 2021), which could be explained by the species-specificity of the effect of CuO NP on NO homeostasis.

Table 1

CuO nanoparticle-induced changes in root length, root number, and lateral root number, and the viability of the apical meristematic zone of sorghum, wheat, rye, and triticale. The results are expressed as the mean \pm s.e. Different letters indicate significant differences according to Duncan's test ($P \leq 0.05$).

Species	Treatment	Root length (mm)		(Lateral) root number (pieces)			Root fresh weight (mg)		Viability - FDA fluorescence (pixel intensity)				
Sorghum	Control	5.67	\pm 0.19	a	3.47	\pm 0.28	f	23.60	\pm 1.68	c	7659.60	\pm 693.72	a
	50 mg/L CuO	2.49	\pm 0.09	de	3.71	\pm 0.21	f	9.96	\pm 0.62	e	3433.03	\pm 293.79	b
Wheat	Control	3.74	\pm 0.18	bc	4.83	\pm 0.09	abcd	48.20	\pm 3.40	b	4508.75	\pm 607.57	b
	150 mg/L CuO	1.96	\pm 0.06	e	4.38	\pm 0.10	cde	16.46	\pm 1.11	de	1154.15	\pm 162.11	c
Rye	Control	4.08	\pm 0.18	b	4.84	\pm 0.21	abc	50.24	\pm 3.93	b	907.45	\pm 120.85	c
	150 mg/L CuO	2.42	\pm 0.08	d	4.69	\pm 0.16	abcde	19.57	\pm 1.68	cd	690.75	\pm 142.14	c
Triticale	Control	4.07	\pm 0.23	bc	5.37	\pm 0.20	a	67.85	\pm 4.51	a	1330.01	\pm 134.53	c
	150 mg/L CuO	2.01	\pm 0.05	e	5.00	\pm 0.15	ab	27.16	\pm 1.99	c	1171.43	\pm 178.72	c

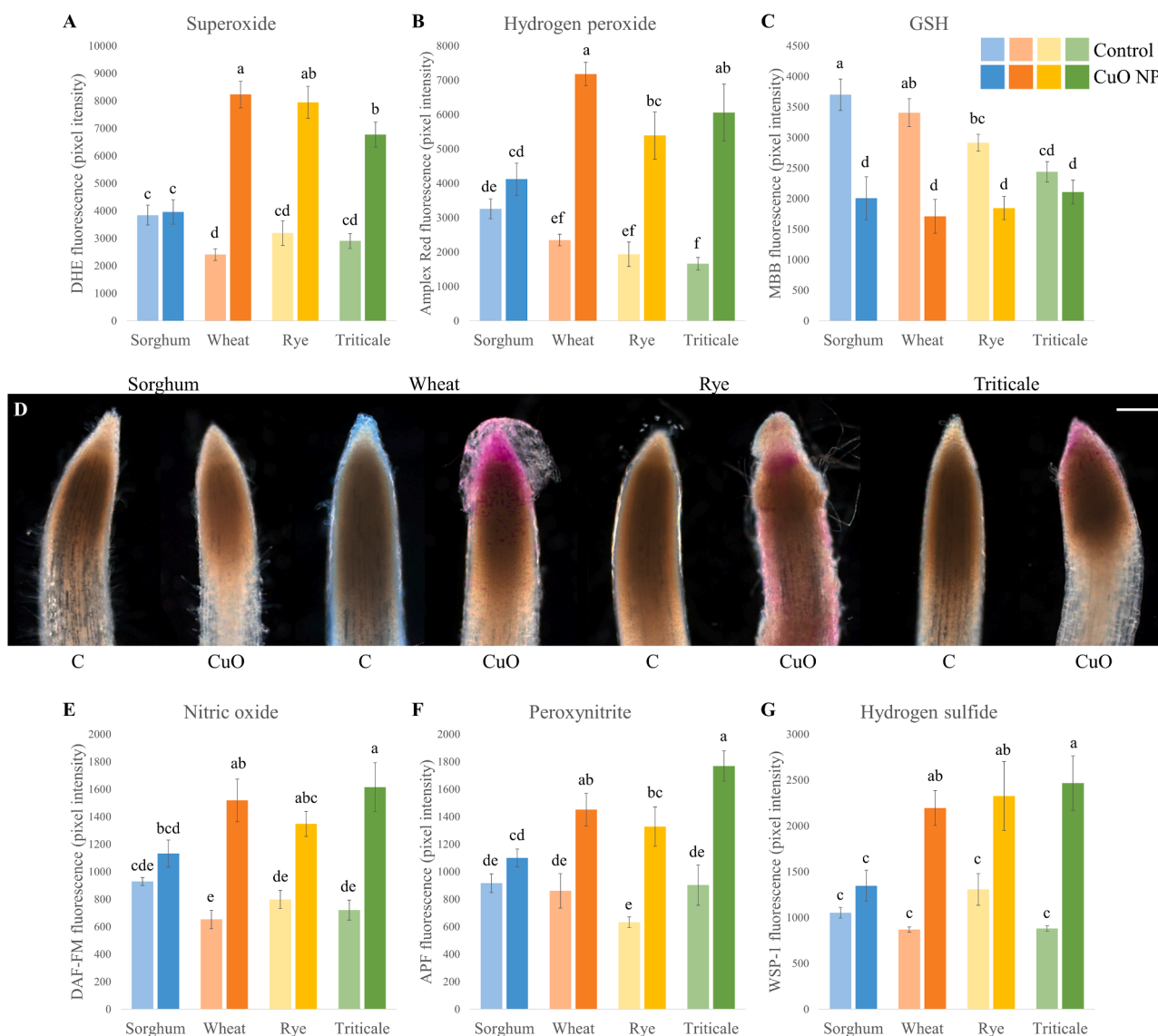


Fig. 2. CuO nanoparticle-induced changes in the levels of reactive molecules and lipid peroxidation in sorghum, wheat, rye, and triticale root tips. Superoxide (A), hydrogen peroxide (B), glutathione (C), malondialdehyde (D), nitric oxide (E), peroxynitrite (F) and hydrogen sulfide (G) content. The results are expressed as the mean \pm s.e. Different letters indicate significant differences according to Duncan's test ($P \leq 0.05$).

Peroxynitrite is formed in the reaction of superoxide and nitric oxide. In the more tolerant species and in correlation with the NO levels discussed above, ONOO⁻ content was significantly increased by CuO NP stress. Moreover, a moderate ONOO⁻ accumulation could also be detected in sorghum root tips (Fig. 2F), which is probably due to a slight increase in the amount of NO.

Hydrogen sulfide is an endogenous gaseous signal molecule in plants that is involved in seed germination (Baudouin et al., 2016) and lateral root formation (Mei et al., 2017) in a similar manner as NO. Moreover, it is able to alleviate different abiotic stressors, such as chromium, cadmium, salinity, and drought (reviewed by Corpas et al., 2019). The observed changes in H₂S content of plant root meristematic zones were

similar to other reactive molecules. In relatively sensitive sorghum, the levels did not change significantly, whereas in the relatively tolerant species, CuO NP stress induced significant H₂S accumulation (Fig. 2G). Increasing evidence has suggested that H₂S is an important signal molecule that has an important effect in the alleviation of heavy metal toxicity in plants and reduced the CuO NP-induced growth inhibition of tomato (Jia et al., 2022). This raises the possibility that the increased endogenous levels of H₂S are responsible, in part, for the relative tolerance of wheat, rye, and triticale.

Based on the changes in the balance of reactive molecules in root tips, despite their similarly inhibited root elongation, the relatively tolerant plant species exhibited a radically different response compared with the relatively sensitive sorghum plant. In the root tips of wheat, rye, and triticale, CuO NP stress induced a significant accumulation of all reactive molecules, regardless of the plant species, despite exhibiting the same morphological response under much lower CuO NP stress, whereas no such response was detected in sorghum.

3.4. CuO NP-induced changes in protein tyrosine nitration in the roots

Despite the fact that CuO NP did not cause a significant change in the amount of reactive molecules in sorghum, the level of protein tyrosine nitration (PTN), a marker of nitro-oxidative stress, increased significantly in CuO NP-treated roots. While PTN is dependent on the presence of peroxynitrite (Corpas et al., 2013), the level does not necessarily show a correlation because its appearance inside the cell can be influenced by a variety of intermolecular processes.

Regardless of the uniform increase in reactive molecules in the relatively tolerant species, CuO NP-induced changes in the nitroproteome exhibited a species-specific response (Fig. 3). In the roots of wheat, nitration levels were unchanged compared with the control and the overall amount of nitrated proteins was low. In the roots of rye, CuO NP increased the nitration of proteins in the size range of 70 and 25 kDa; moreover, several new immunopositive bands appeared in the proteome of the stressed root (50, 35, and 23 kDa). In triticale, several nitrated protein bands were visible in the control samples (70, 35, 25, and 23 kDa), but with the exception of the 70 kDa band, the nitration signal decreased under CuO NP stress. Although showing similar growth response accompanied by analogous changes in the homeostasis of the reactive molecules in their roots, the relative tolerant species exhibited three different nitration responses. It is also important to note that the species-specific nitration response was significantly less pronounced compared with the large increase in nitration resulting from CuO NP

stress in the relatively sensitive sorghum root. Nitrated proteins exist in non-stressed plants as well and the physiological nitroproteome has been detected in a number of species (Chaki et al., 2009; Kolbert et al., 2017). Based on previous studies with heavy metals, nickel-induced changes in PTN increased to a smaller degree in the more tolerant *Brassica juncea* compared with the relatively sensitive *Arabidopsis thaliana* (Kolbert et al., 2020). In *Brassica napus*, a similar stress level-dependent change was observed in PTN. Growth-inducing, low concentration zinc treatment resulted in a change in the nitration pattern of the root, whereas stress-inducing, high concentrations of zinc significantly increased nitration in the roots (Feigl et al., 2019). In another study, combined heavy metal stress resulted in distinct changes in PTN in the relative tolerant *B. napus* and sensitive *Helianthus annuus*. The tolerance to heavy metals was evident by a changed pattern of PTN, whereas the stress state of the root was accompanied by increased levels of PTN (Feigl et al., 2020).

Similar to triticale, in the selenium hyperaccumulator, tolerant *Astragalus bisulcatus* root PTN decreased under selenium stress, whereas in the sensitive *A. membranaceus*, the degree of PTN significantly increased (Kolbert et al., 2018). The underlying mechanisms that causes decreased PTN have not been elucidated; however, the degradation of proteins via proteasomes as a tolerance response to excess stress negatively affected protein content, including nitrated proteins, to reverse the adverse effects to the proteome (Kolbert et al., 2018; Castillo et al., 2015; Van Hoewyk et al., 2018; Molnár et al., 2020a).

Our results are consistent with the above findings, in which the relative sensitivity of plant species to (heavy) metal stress is evidenced by increased PTN, whereas relative tolerance is accompanied by less severe changes in nitration levels, which often includes changes in nitration pattern and decreased PTN.

3.5. CuO NP-induced changes in the cell wall-mediated defense system

Plants are able to protect their cells by actively change the structure and chemical composition of their cell walls (Houston et al., 2016). CuO NP stress induced various modifications in the root tip cell wall of the relatively sensitive sorghum plant compared with that in other tolerant species. Of the four species, CuO NP caused lignin encrustation only in the root tips of the sorghum plant (Fig. 4A). In contrast, callose accumulation was not observed, only in the more tolerant species (Fig. 4B).

The amount of quercetin, a flavonoid component of the cell wall-related defense machinery, was only increased during CuO NP-induced stress in the root tip of wheat (Fig. 4C). Compared with the

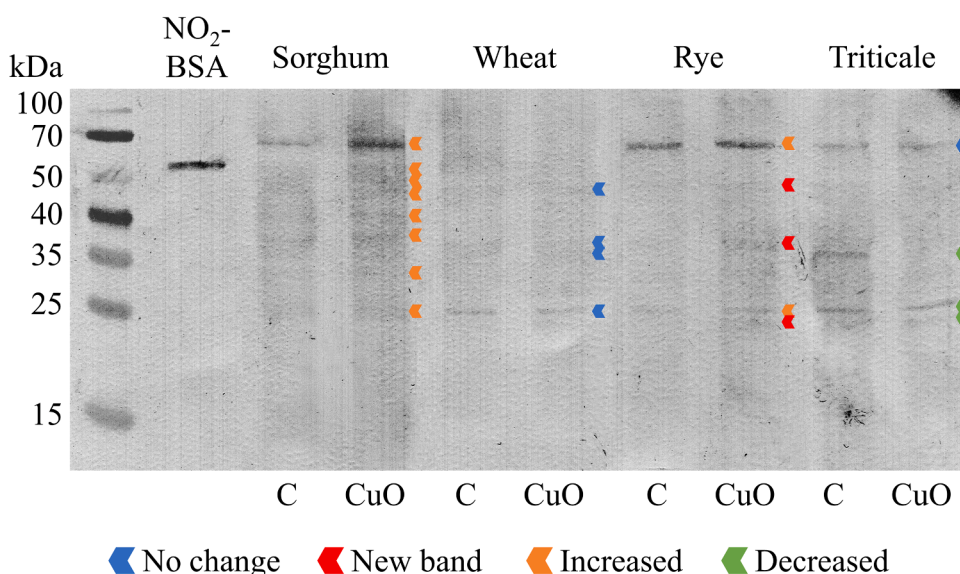


Fig. 3. Representative immunoblot indicating protein tyrosine nitration in the roots of sorghum, wheat, rye, and triticale under control conditions and subjected to CuO NP stress. Blue arrows show nitrated protein bands with no change compared with the control, red arrows indicate new nitrated bands, orange arrows represent protein bands with increased nitration compared with the control, green arrows indicate protein bands with decreased nitration compared with the control. (NO₂-BSA: nitrated bovine serum albumin, used as positive control).

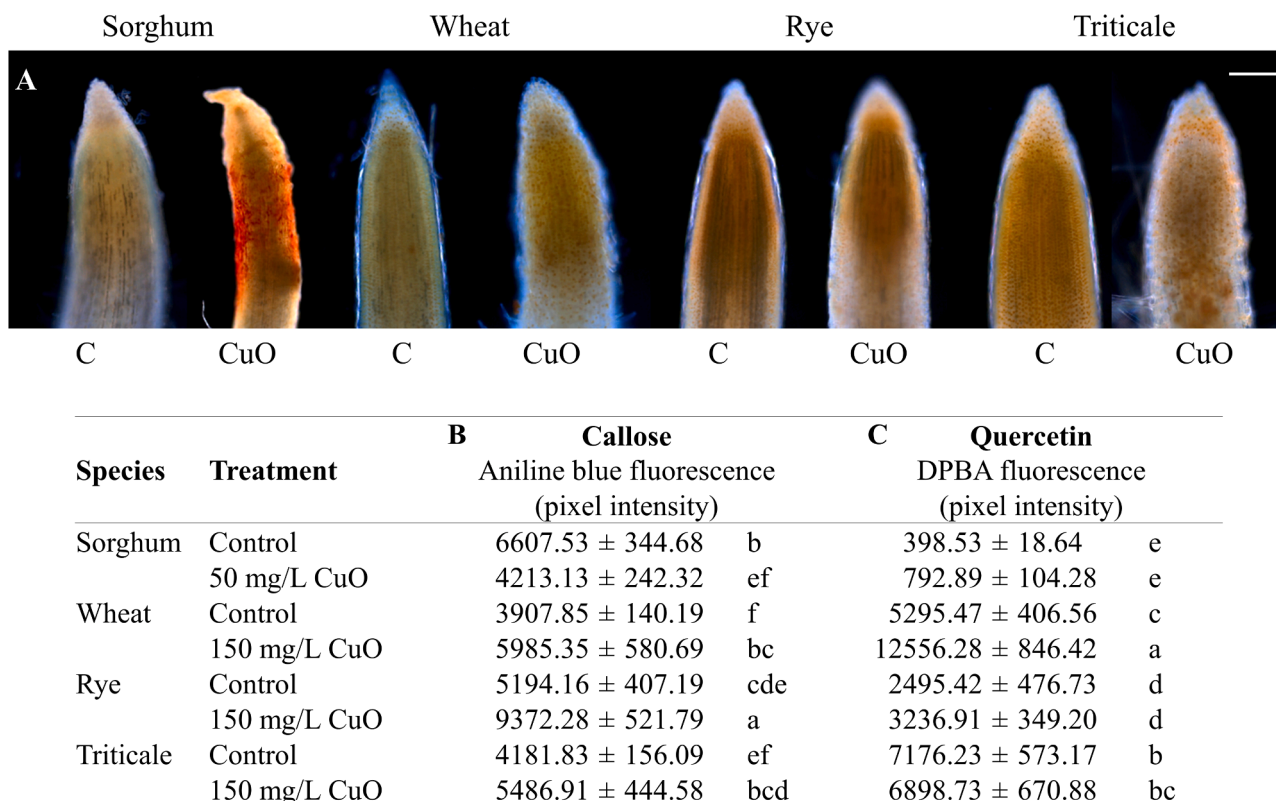


Fig. 4. CuO nanoparticle stress-induced cell wall-mediated defenses. Lignin (A), callose (B), and quercetin (C) content of the root tips of sorghum, wheat, rye, and triticale. The results are expressed as the mean ± s.e. Different letters indicate significant differences according to Duncan's test ($P \leq 0.05$).

more CuO NP-tolerant species, the quercetin content of sorghum root tips was significantly lower and not increased by CuO NP stress. Exogenously added quercetin prevented PTN in rat neuronal precursor cells (Sajad et al., 2013); however, no similar experiment has been conducted in plants. Nevertheless, the amount of endogenous quercetin detectable in the root apex was inversely proportional to the degree of nitration, suggesting a possible connection between the two. In sorghum, in which the least amount of quercetin was detected in the roots, nitration increased significantly following CuO NP treatment, whereas in wheat, a significant increase in quercetin content was observed and the level of nitration did not change during CuO NP stress. In rye and triticale, CuO NP stress did not induce quercetin accumulation; however, in rye, the quercetin content was significantly lower compared with triticale (or wheat), protein nitration was increased, and a higher amount of quercetin was accompanied by decreased PTN levels.

4. Conclusions

Because of their beneficial properties, copper oxide nanoparticles are becoming increasingly important in industry and agriculture; however, only a few studies have examined their effects on plants. The present study is the first to examine the processes behind the growth inhibitory effect of CuO NPs through a comparison of four economically important agricultural monocotyledonous plants.

Although the four examined species showed a similar growth response to CuO NP stress, different amounts were required to achieve 50% root length inhibition. With respect to the relative CuO NP sensitivity of sorghum roots, the balance of reactive molecules was not significantly disturbed. Moreover, PTN and lignin content of the cell walls increased considerably, and neither lipid peroxidation nor callose deposition in the root tips was detectable.

In the relative CuO NP-tolerant wheat, rye, and triticale, the amount of reactive molecules increased significantly and was accompanied by

increased lipid peroxidation and callose content in the root tips. The relative tolerance of the roots from these species is evidenced by the different CuO NP-induced changes in PTN, which exhibited a negative correlation with the quercetin content of the root tips. It is also possible that the increased endogenous levels of H_2S in the roots of wheat, rye, and triticale are at least partly responsible for their relative tolerance compared with sorghum.

The results of this study indicate that although different monocotyledonous species with different CuO NP sensitivities exhibit similar growth responses, diverse changes may occur in the balance of reactive molecules in their roots, which can indicate and influence their tolerance to CuO NPs as a stressor.

Author contributions

G.F. designed the research; B.K., E.M., Á.Sz., A.R. and G.F. performed research; G.F. supervised the experiments, analyzed the data, and wrote the draft version; A.R., Z.K. and G.F. reviewed and edited the final manuscript.

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.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2023.100145.

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