

Copper oxide nanoparticle effects on root growth and hydraulic conductivity of two vegetable crops

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Abstract

Aims Root growth and water transport were evaluated for two vegetable crops of contrasting root architecture (lettuce, carrot) exposed to copper oxide nanoparticles (CuO NPs).

Methods 10-day seedling root growth assays were evaluated for 16 nanometer (nm) diameter CuO NP and CuCl₂ control (0.8 – 798.9 mg Cu L⁻¹). In a separate experiment, hydraulic conductivity (K_h) of root systems not previously exposed to NP was tested using 16 and 45 nm CuO NP (798.9 mg Cu L⁻¹) relative to CuO NP-free controls, and xylem sap was assessed by TEM-EDS for presence of CuO NPs.

Results 16 nm CuO NP produced dose-dependent increases in root diameter for lettuce (+52%) and carrot (+26%) seedlings, whereas CuCl₂ did not affect (lettuce) or marginally increased (carrot) root diameter. Root K_h was similarly reduced by 16 and 45 nm CuO NPs for lettuce (-46%) but not for carrot, and no Cu was identified by TEM-EDS in xylem sap.

Conclusions Adverse effects of CuO NPs on root physiology and function in the early stages of growth of two key food crops are not necessarily due to Cu²⁺ toxicity and can be specific to crop species. In addition to triggering root thickening,

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reduction of root K_h signifies that CuO NPs can compromise root water transport and thus crop performance.

Keywords Copper oxide · Nanoparticles · Roots · Hydraulic conductivity · Lettuce · Carrot

Abbreviations

CuO copper oxide

NP nanoparticle

K_h hydraulic conductivity

Introduction

The recent proliferation of engineered nanoparticles (NPs) entails greater exposure of organisms in agroecosystems via recycled waste streams, accidental contamination events, and intentional inputs (Parisi et al. 2015; Sekhon 2014), potentially impacting the growth of crop species. The unique properties of materials in NP forms (<100 nm in all dimensions), such as high specific surface area, raise the possibility of environmental effects not observed for other forms of the same material (Nel et al. 2006).

Copper oxide (CuO) NPs are emerging as a next-generation of Cu fungicides (Elmer and White 2016; Giannousi et al. 2013; Li et al. 2017), which have been in widespread use in perennial and annual crops since 1761 (Eduok and Coulon 2017). Though foliar applied, including CuO NP (Giannousi et al. 2013; Li et al. 2017), downward movement of Cu fungicides to soil inevitably occurs as a result of washing by irrigation and precipitation, as well as via drift and accidental application to soil, resulting in exposure of root systems to Cu (Chaignon et al. 2003). Additional exposure of food crop root systems to CuO NP could occur via waste streams, in particular with the increasing use of recycled wastewater for irrigation and the expansion of hydroponic agricultural (Brar et al. 2010; Sun et al. 2014).

NPs have potential to impact the growth of food crops directly by inhibiting root growth and indirectly by compromising root functions such as water transport. Compared to other metal oxide NPs (e.g., TiO_2 , Fe_2O_3), CuO NPs have consistently shown negative effects on the growth of a variety of aquatic and terrestrial plant species, including food crops (Du et al. 2016). Previous investigations of CuO NP effects on plant growth found greater effects on roots than shoots (e.g., elongation,

thickness, physiological and cellular damage), mediated largely through metal toxicity of dissolved Cu^{2+} . In contrast to the conspicuous molecular and cellular damage inflicted by metal ion or ‘chemical toxicity’, negative effects of NPs on plants may be mediated through more subtle physical mechanisms (Servin and White 2016). For example, exposure of root systems to NPs could compromise root functions such as water transport, with likely impacts on plant growth and productivity. However, evaluations of metal oxide NPs for such ‘physical toxicity’ on root functions are limited (Asli and Neumann 2009).

Root hydraulic conductivity is a measure of the efficiency with which water moves into and through a root system and provides an estimate of its capacity to supply water to a transpiring canopy. As such, it is a fundamental indicator of plant function and performance, is linked to photosynthetic and growth capacity, and is highly sensitive to environmental changes (Melcher et al. 2012). Exposure of root systems to NPs could inhibit water uptake capacity of roots by blocking cell wall pores (Asli and Neumann 2009). Such responses need further investigation across types of NPs and plant species, in particular CuO NP given its potential use in agricultural production systems (Giannousi et al. 2013).

We investigated potential impacts of CuO NP on the root physiology and root function of two food crop species of contrasting root architecture, lettuce and carrot. The objectives of the present work were to 1) evaluate the response of early root growth (i.e., elongation and diameter), including germination, to CuO NP exposure relative to a Cu^{2+} control, 2) assess physical effects of CuO NP on lettuce and carrot root function by quantifying hydraulic conductivity (K_h) of root systems, and 3) determine whether CuO NPs are able to pass through the root tissue and into the xylem for long distance transport to the canopy during acute exposure in the previous objective.

Materials and methods

CuO NP synthesis and characterization

For germination and root growth experiments, CuO NPs were obtained by laboratory synthesis. Wet precipitation was used to synthesize 16 nm diameter CuO NP (Siddiqui et al. 2013). Briefly, 300 mL of 0.2 mol L^{-1} copper (II) acetate monohydrate [$Cu(CH_3COO)_2 \cdot H_2O$] and 1 mL of glacial acetic acid were heated and vigorously mixed in

an Erlenmeyer flask capped with a watch glass. Upon boiling, 15 mL of 6 mol L⁻¹ sodium hydroxide solution was added to induce rapid formation of CuO NPs. The resulting precipitate was concentrated by centrifugation (60 min at 15,000 RCF), washed repeatedly on sterile 0.22 µm filter paper with 18.2 MΩ·cm water (Barnstead NANOpure, Thermo Fisher Scientific, USA) and 95% ethanol, and dried at 60 °C for 6 h.

Two sizes of CuO NPs were selected (16 and 45 nm diameter) in order to bound the maximum cell wall pore diameter of 20 nm proposed to restrict direct entry of NP into apoplastic pathways (associated with the hydraulic conductivity measurements described below) (Hatami et al. 2016). The 16 nm CuO NPs were obtained by synthesis as described above and the 45 nm diameter CuO NP were purchased from Sigma Aldrich (USA). Primary particle size and shape of CuO NPs were characterized by transmission electron microscopy (TEM) at 120 kV (Philips CM-12, The Netherlands). Samples were prepared for TEM analysis by placing 10 µL of a stock of 100 mg L⁻¹ CuO NP suspended in ethanol on formvar film coated Cu grids (Ted Pella, USA) and drying the grids over an incandescent bulb. Mean primary particle size ($n > 200$) for both CuO NP sources was calculated from TEM images using Fiji (Fig. S1) (Schindelin et al. 2012). Synthesized CuO NP had a diameter of 16.0 ± 10.0 nm and commercially available CuO NP had a diameter of 45.2 ± 11.2 nm (advertised as 50 nm). Particle mineralogy was confirmed by X-ray diffraction using a Cu X-ray source operating at a tube voltage of 40 kV, a tube current of 40 mA, and a scan rate of $2^\circ 2\theta \text{ min}^{-1}$ (Rigaku Ultima IV, Japan) (Fig. S1c). Mineral identification was performed using Jade 9 (MDI, Livermore, USA).

Food crop species

Lettuce (*Lactuca sativa*, cv. Nevada Summer Crisp) and carrot (*Daucus carota* subsp. *sativus* cv. Little Finger) were purchased from Swallowtail Garden Seeds (Santa Rosa, CA) and Botanical Interests (Boulder, CO), respectively. These two species were selected due to differences in root morphology and physiology and for their use as food crops differing in the edibility of above-ground (leaf) versus below-ground (root) biomass.

Root growth of seedlings

Root elongation and root thickness (i.e., diameter) of lettuce and carrot seedlings were assessed over a 10-day

germination and growth period. Three experimental treatments for each crop species included: DI water (negative control), 16 nm diameter CuO NPs, and Cu²⁺ as CuCl₂. CuO NP treatments of 1, 10, 50, 100, 500, and 1000 mg L⁻¹ were included, which on a Cu basis corresponded to 0.8, 8.0, 40.0 (carrot only), 79.9, 399.5, 798.9 mg L⁻¹. A parallel series of CuO NP treatments without seeds were measured over the growth period (0, 3, 6, 12, 24, 48, 96, 168, 240 h) to quantify Cu²⁺ dissolution from CuO NPs and enable comparison with the Cu²⁺ control. In order to distinguish CuO NP toxicity from dissolved Cu²⁺ toxicity, Cu²⁺ treatments in the form of CuCl₂ were used to bound Cu²⁺ concentrations resulting from dissolution of CuO NPs during the 10-day growth assay (McShane et al. 2014). Previous assessments of CuO NP generally use a single concentration of an ionic Cu control such as CuCl₂ to bound the upper limit of Cu²⁺ thought to dissolve from CuO NPs (e.g., Wang et al. 2012). However, we employed a range of CuCl₂ concentrations to enable greater comparisons with Cu²⁺ solubilized from CuO NP (Rippner et al. 2018) and to evaluate potential concentration-dependent CuCl₂ effects on lettuce and carrot root growth and length. CuCl₂ treatments of 0.1, 1, 10, 50, and 100 mg L⁻¹ were used to establish Cu-basis treatments of 0.1, 0.5, 2.4 (carrot only), 4.7, 23.6 (lettuce only), and 47.3 mg L⁻¹. These range of CuCl₂ concentrations were selected to bound Cu²⁺ concentrations reported to result from dissolution of CuO NPs.

For each species, germination rates at days 7 and 10 (168 and 240 h) were assessed using 10 seeds for each treatment level. Seeds were placed on moist (18.2 MΩ·cm water) cellulose filter paper (Atha et al. 2012; Dimkpa et al. 2015; Ko and Kong 2014; Yang and Watts 2005) with equal spacing in petri dishes with 7 mL of treatments using replicate dishes ($n=3$) for each treatment level, and incubated under an 18-6 h day-night cycle at 22 °C. The presence of a root radicle was deemed positive germination. Mean root length and diameter of each replicate (i.e., petri dish with 10 seeds) were measured using WinRhizo (Regent Instruments, Quebec City, Canada; resolution of 0.005 mm) at days 7 and 10.

Hydraulic conductivity (plant physiological function)

To determine CuO NP impacts on root water uptake capacity, root system hydraulic conductivity (K_h) measurements were performed using 21-28 day-old lettuce

and carrot plants. Plants were germinated in vermiculite and transplanted into the hydroponic chambers 14–20 days after germination. Vermiculite was removed from seedlings by washing with 18.2 M Ω ·cm water prior to transplanting in hydroponic chambers. Plants were grown hydroponically in $\frac{1}{4}$ Hoagland solution, using fresh Hoagland solution every 5–7 days, under an 18–6 h day–night cycle at 22 °C. Solution aeration was maintained by bubbling air from the bottom of the chamber (Norén et al. 2004). To prevent algae growth, the bottom portion of the chambers were kept dark by wrapping the exterior in opaque aluminum foil. Measurements of root K_h were performed when plants had sufficiently large root systems and stem thickness (21–28 days) to allow measurement procedures without tissue damage. This approach provides the additional advantage of avoiding confounding effects of chemical toxicity during the preceeding plant growth stage in order to isolate a potential physical effect of NPs on root water transport.

To test the hypothesized physical inhibition effect of NPs on root water transport, root K_h response to CuO NPs was evaluated relative to the same CuO NP-free Hoagland solution used to grow plants prior to performing K_h measurements (Asli and Neumann 2009; Martínez-Fernández et al. 2016; Martínez-Fernández and Komárek 2016). An additional control of deionized water was used to account for potential solute effects of Hoagland solution (Asli and Neumann 2009). Two CuO NP were tested with diameters of 16 and 45 nm that bound the threshold cell wall pore diameter of 20 nm thought to restrict direct entry of NP into apoplastic pathways (Davis et al. 2017; Hatami et al. 2016), with root systems exposed to a solution of 798.9 mg Cu L⁻¹. This CuO NP exposure concentration was chosen because 1) it bounds the concentration gradient used in root growth assays, 2) hypothesized physical effects of NPs on root water transport (i.e., pore shielding or clogging) are likely to occur at NP concentrations higher than the concentrations at which chemical toxicity effects occur (Asli and Neumann 2009), 3) it represents conceivable exposure hotspots that occur during washing off of foliar-applied Cu fungicides onto root systems (Pérez-Rodríguez et al. 2013), and 4) it is in the range of NP concentrations reported for accidental spill scenarios or contaminated wastewater used for irrigation (Servin and White 2016).

Root K_h was measured by fitting excised stems with a pipette to measure and capture xylem sap outflow. The root system was fully submerged into a solution, which differed by treatment (i.e., 16 nm CuO NP, 45 nm CuO

NP, Hoagland solution, or deionized water), enclosed in a pressure chamber (PMS Instruments, USA) and immediately subjected to a series of increasing pressures (0, 0.069, 0.10, 0.15, 0.20, 0.25 MPa) over 2 h. For each pressure (P), the volume (V) of xylem effluent was measured. The flow rate (Q) was calculated as using the linear regression of V versus ΔP , from which K_h was determined as the slope. The response curves (V as a function of P) were linear across this pressure range.

For each plant individual, two sets of flow measurements were performed. First, de-ionized water was used to perform an initial K_h measurement, followed by a 20 min rest period. This initial flow-through can flush potential embolisms introduced by excision (Tyree et al. 1992). Flow was then measured using one of four treatments: (1) deionized water; (2) 16 nm CuO NP; (3) 45 nm CuO; and (4) $\frac{1}{4}$ Hoagland solution. Time was recorded for a target sap collection of 40 μ L for lettuce and 20–30 μ L for carrot.

To evaluate the potential vascular transport of CuO NP from root to shoot systems during acute NP exposure, xylem sap harvested during K_h measurements was examined by scanning transmission electron microscopy (STEM). First, 20 μ L of sap from each plant individual was dried on carbon type-B film coated gold TEM grinds (Ted Pella, USA) and imaged in triplicate with STEM at 20 kV with a backscatter detector (FEI XL30, USA). Energy dispersive x-ray spectroscopy (EDS) was performed using a Genesis EDS detector and spectra analyzed with Genesis software (EDAX, USA). To inform visual identification of CuO NPs and interpretation of ED spectra, a subsample of harvested sap was spiked with 5 μ L of 16 nm diameter CuO NP (100 mg L⁻¹). To ensure thorough spectroscopic evaluation of potential transport, xylem sap solids were concentrated by centrifugation at 21,000 RCF for 30 min and additional imaging and analysis was performed using STEM and EDS.

Statistical analyses

All statistical analyses were performed separately for lettuce and carrot. Assumptions of normality and homoscedasticity of residuals for response variables were evaluated using Shapiro-Wilk's test and Levene's test, respectively. Treatment effects on 10-day germination, root length, and root diameter were assessed by one-way analysis of variance (ANOVA) and post-hoc mean differences were determined by Tukey's test ($p < 0.05$) using PROC GLM with SAS v9.4 (SAS Institute, Cary, NC).

The F-statistic was used to compare the relative magnitude of Cu effects on a given plant response variable between lettuce and carrot. Differences in Cu^{2+} dissolution between days 7 and 10 were evaluated by pairwise t-test (two-sided, $\alpha = 0.05$). Treatment effects on root K_h were evaluated using a mixed model with PROC MIXED to account for differences in replication in K_h measurements. K_h values were log transformed in order to meet assumptions of residual normality and homoscedascity. Post-hoc analysis of mean differences K_h were determined by least square means using the Tukey-Kramer adjusted test for multiple pairwise comparisons.

Results

Cu^{2+} dissolution from 16 nm CuO NP

Over the course of the 10-day period used to assay germination and root growth, dissolution of 16 nm diameter CuO NP approached equilibrium of aqueous Cu^{2+} within 48 h (Fig. 1). Though dissolution of CuO NP (8–71%) decreased with increasing concentration of CuO NPs (Fig. S2), larger differences in treatment concentrations of Cu as CuO NP resulted in distinct concentrations of Cu^{2+} that increased with initial CuO NP concentration (Fig. 1). At day 10, these corresponded to 0.6, 1.6, 4.7, 10.7, 39.7, and 66.8 mg Cu L^{-1} for CuO NP treatments of 0.8, 8.0, 40.0, 79.9, 399.5, 798.9 mg Cu L^{-1} , respectively. For a given CuO NP concentration, Cu^{2+} concentrations at days 7 and 10 (168 and 240 h) did not differ except for 798.9 mg Cu

L^{-1} , which was lower ($p = 0.031$) at day 10 ($66.8 \pm 1.2 \text{ mg L}^{-1}$) than at day 7 ($80.0 \pm 2.9 \text{ mg L}^{-1}$).

Plant germination and growth

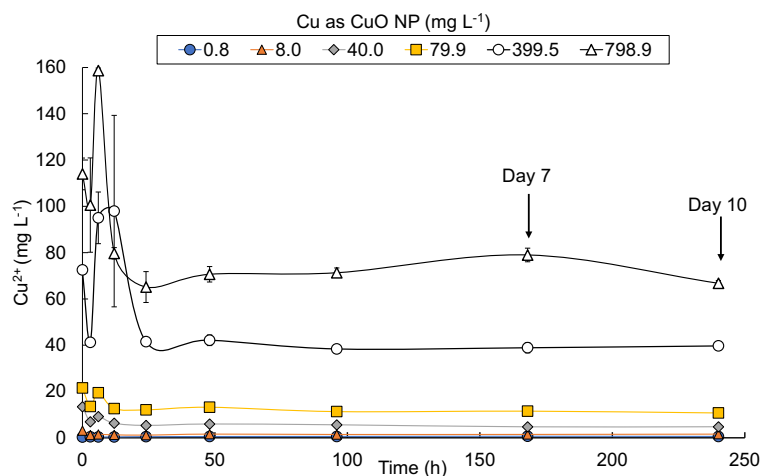
CuO NPs had no significant effect on germination of lettuce and carrot across four magnitudes of CuO NP concentrations measured (0.8–798.9 mg L^{-1} Cu as CuO NP) (Fig. 2a, b) despite producing Cu^{2+} concentrations similar to CuCl_2 (Fig. 1). Carrot germination tended to be lower, albeit non-significantly, at the intermediate CuO NP concentration of 40.0 mg Cu L^{-1} (0.39) compared to the control (0.93).

In contrast, Cu in the form of CuCl_2 decreased germination of both food crop species at concentrations $\geq 4.7 \text{ mg Cu L}^{-1}$ (Fig. 2c, d). Carrot germination was impacted more than that of lettuce ($F = 44.6$ vs 23.3), decreasing by 86% at the highest Cu treatment of 47.3 mg Cu L^{-1} . Lettuce germination was reduced by a maximum of 50% at the highest CuCl_2 treatment.

Root length of seedlings decreased with increasing concentration of CuO NP and CuCl_2 in a similar manner for both species; roots were shorter under CuCl_2 than for CuO NP (Fig. 3). CuO NP had a greater effect on root length for lettuce ($F = 23.5$) than carrot ($F = 5.4$). Reduction of lettuce root length by CuO NP (–54%) at 8.0 mg Cu L^{-1} did not differ with two orders of magnitude greater exposure. Carrot root length exhibited a similar trend of halved length at CuO NP concentrations $\geq 8.0 \text{ mg Cu L}^{-1}$, though length reduction was only significant for 40.0 mg Cu L^{-1} (–47%).

Fig. 1 CuO NP dissolution.

Dissolution of 16 nm diameter CuO NP over the course of 10 days was measured as cupric ion (Cu^{2+}). Error bars represent standard error. Arrows indicate measurement of germination rate, root length, and root diameter at days 7 and 10. Lines are meant as a guide among measurements taken at varying time points, and do not represent data



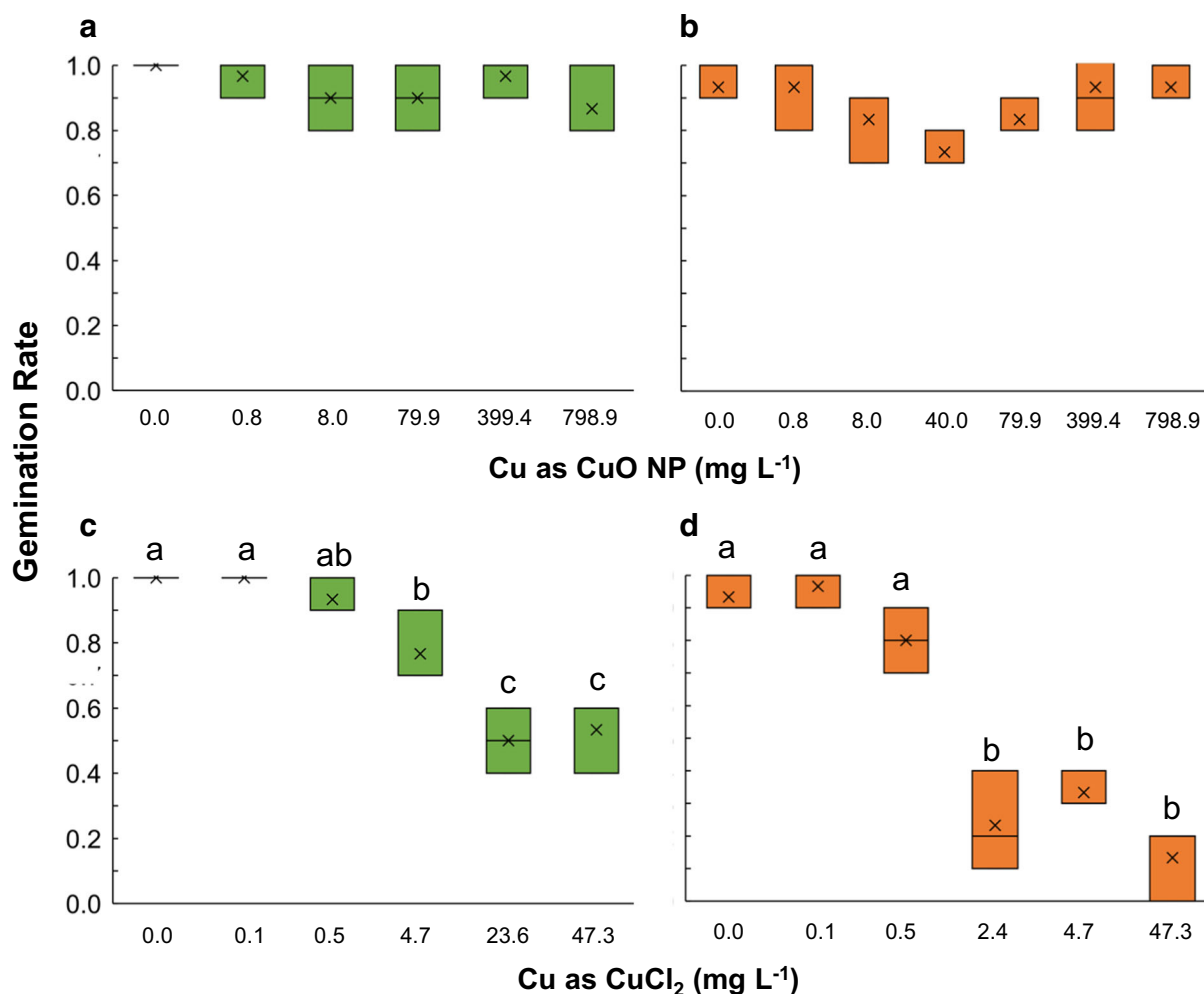


Fig. 2 CuO NP and germination. Germination of lettuce (green) and carrot (orange) seeds after 10-day exposure to Cu in the form of (a, b) CuO NPs (16 nm diameter) and (c, d) CuCl₂. Box plots depict mean (x) and median (solid line). Different letters represent

significant differences within a given species determined by Tukey's test ($p < 0.05$). The absence of letters indicates the absence of significant differences among means

Similar reductions in root length occurred for lettuce and carrot with CuCl₂ at concentrations of 0.5 mg L⁻¹ and greater. Cu exposure in the form of CuCl₂ had a greater effect than in the form of CuO NP across the tested concentrations (e.g., lettuce $F_{\text{CuCl}_2} = 437.1$ vs $F_{\text{CuO NP}} = 5.4$). CuCl₂ at concentrations of 4.7 mg L⁻¹ and greater strongly inhibited root growth in lettuce (-94%) and carrot (-99%), and fully inhibited germination for carrot at 47.3 mg Cu L⁻¹.

CuO NP triggered dose-dependent root thickening that was highly similar for lettuce and carrot, but CuCl₂ effects were species-specific (Fig. 4). CuO NP exposure had nearly twice the effect on root diameter for lettuce ($F = 37.5$) than for carrot ($F = 18.9$), with linear increases in

root diameter of up to 52% and 26%, respectively. In contrast, lettuce root diameter was not affected by exposure to CuCl₂ whereas carrot root diameter increased by 56% only at 2.4 mg Cu L⁻¹, with greater mean diameter (0.56 cm) than for carrot exposed to the highest concentrations of CuO NPs (0.49 cm). Root diameter measurements were not possible for carrot at the highest CuCl₂ concentration evaluate due to lack of germination.

Root hydraulic conductivity (K_h)

Hydraulic conductivity of lettuce and carrot root systems was reduced in acute exposure to CuO NP

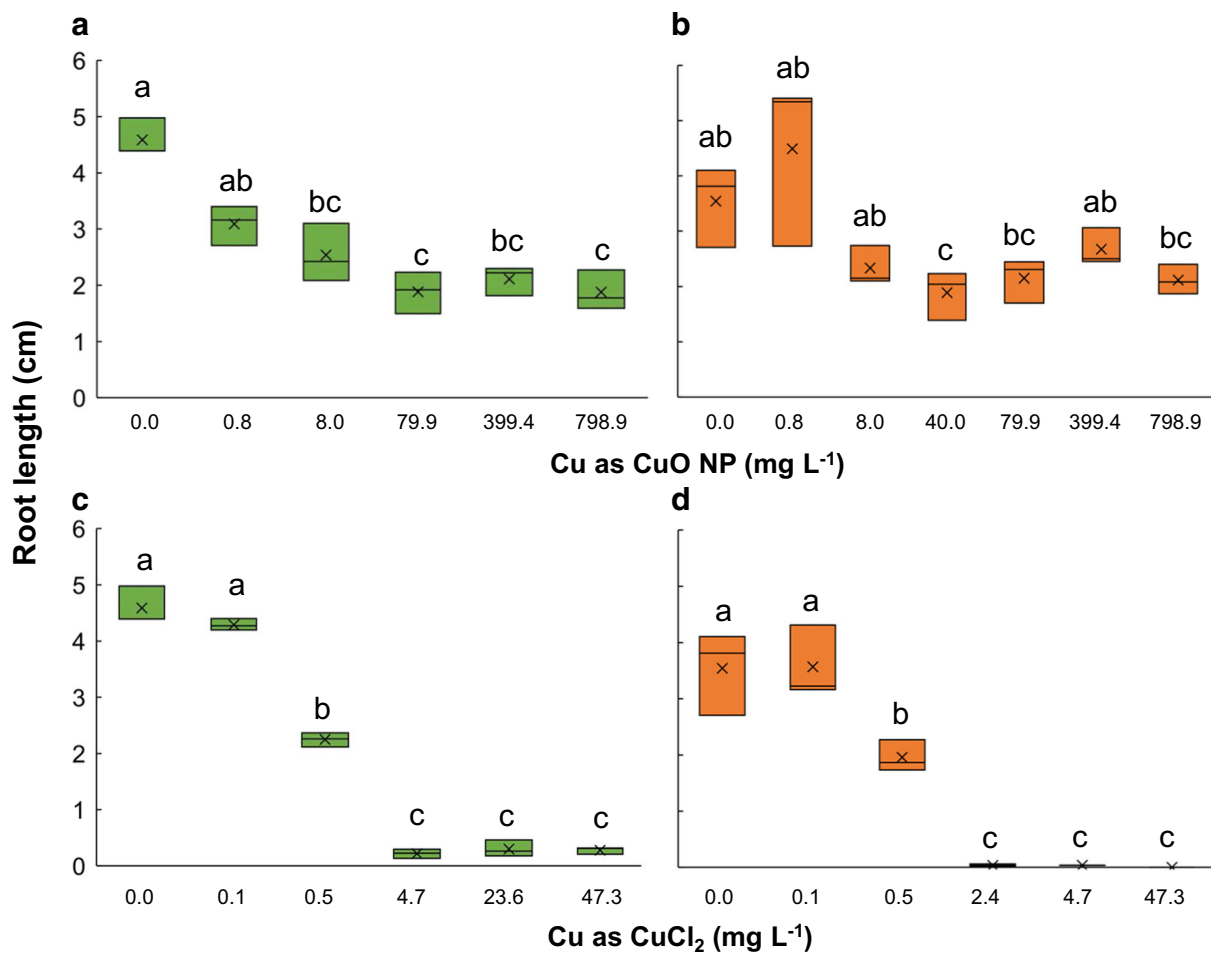


Fig. 3 CuO NP and root length. Root length of 10-day lettuce (green) and carrot (orange) seedlings exposed to Cu in the form of (a, c) CuO NPs (16 nm diameter) and (b, d) CuCl₂. Box plots

depict mean (x) and median (solid line). Different letters represent significant differences within a given species determined by Tukey's test ($p < 0.05$)

(798.9 mg Cu L⁻¹) (Fig. 5). The size of CuO NPs (16 nm and 45 nm diameter) had no effect on root K_h , which for lettuce was reduced by 41% relative to water and Hoagland solution (CuO NP-free) controls. Root K_h of both species was similar for deionized water and Hoagland solution. The K_h of carrot root systems tended to decrease with CuO NP exposure relative to water and Hoagland solution controls ($p = 0.09$).

STEM-EDS observation of xylem sap collected during K_h measurements did not identify the presence of CuO NP or other Cu species (Fig. S3). CuO NPs were not observed in the solid concentrate (centrifugation) of xylem sap combined from replicate root systems (Fig. S3a-b). Sap samples spiked with 100 mg L⁻¹ 16 nm diameter CuO NP clearly showed the expected morphology and ED spectra of CuO NPs (Fig. S3c-d).

Discussion

Nanosize effects on lettuce and carrot germination

Germination of lettuce and carrot seed were not affected by CuO NPs despite significant inhibition of germination by Cu²⁺ as CuCl₂ at Cu²⁺ concentrations measured for CuO NPs. This indicates a non-ion effect of CuO NPs opposite to what is generally observed: Cu²⁺ produced via dissolution of CuO NP did not entail reduced germination, in contrast to similar concentrations of Cu²⁺ from a copper salt. In contrast, several studies indicate that dissolution of ions from metal oxide NPs can inhibit seed germination, including CuO NP (Dimkpa et al. 2012; Shaw and Hossain 2013; Stampoulis et al. 2009; Tang et al. 2013). Possible explanations for this

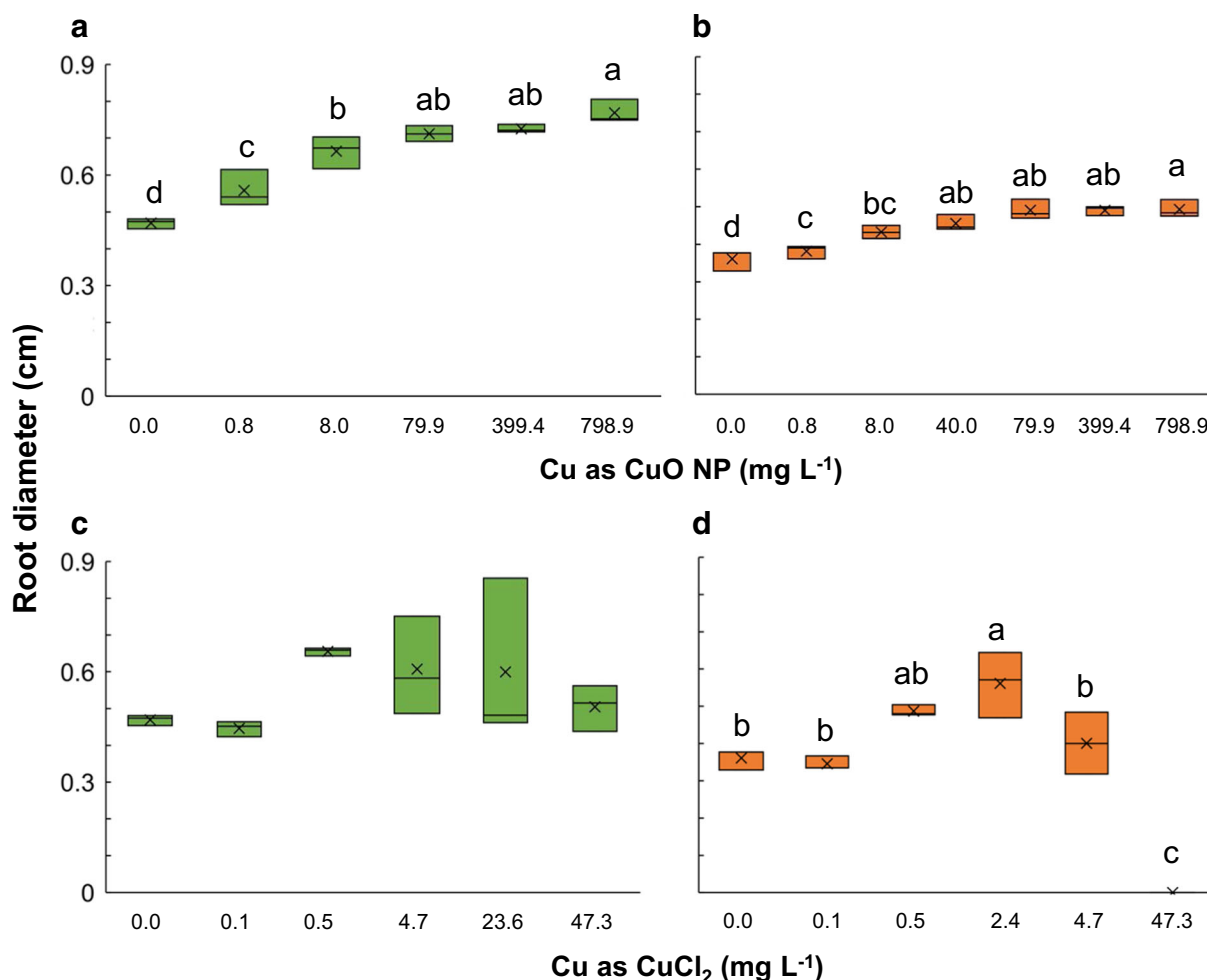


Fig. 4 CuO NP and root diameter. Root diameter of 10-day lettuce (green) and carrot (orange) seedlings exposed to Cu in the form of (a, c) CuO NPs (16 nm diameter) and (b, d) CuCl₂. Box

plots depict mean (x) and median (solid line). Different letters represent significant differences determined by Tukey's test ($p < 0.05$)

discrepancy include 1) interaction of CuO NPs and dissolved Cu²⁺ and 2) toxicity of Cl⁻ in CuCl₂ treatments. Binding of CuO NPs to seed surfaces may have decreased Cu²⁺ effects. The chloride ion in the Cu²⁺ salt treatment may have contributed to inhibit germination, in particular for a sensitive species such as lettuce (Bernard Tinker et al. 1977; Maas and Hoffman 1976). However, we observed decreased lettuce germination for CuCl₂ treatments corresponding to 0.2 – 1.8 mM Cl⁻, which is substantially lower than the 10 – 30 mM Cl⁻ (as NaCl) reported by others to inhibit lettuce germination and growth (Moon et al. 2014). Future NP studies should evaluate additional ionic Cu controls, also referred to as 'salt' or 'soluble' Cu controls (Thwala et al. 2016), in order to isolate the Cu²⁺ effect from potential counter anion effects (e.g., CuSO₄). Increasing concentration of

Cu²⁺ with CuO NP concentration may therefore not necessarily entail greater inhibition of germination via Cu²⁺ toxicity with increasing CuO NP exposure. Finally, we note the importance of quantifying CuO NP dissolution for individual concentrations rather than assuming a dissolution percentage because the percent dissolution of the studied CuO NPs was found to be inverse to concentration (Fig. S2).

Lack of germination response to CuO NP exposure in our study is consistent with suggestions that seed germination is a relatively insensitive measure of NP toxicity compared to root growth because of the short exposure time and protective seed coat. However, such studies used large- to moderately-seeded species such as maize (Wang et al. 2012) and cucumber (Stampoulis et al. 2009) whereas small-seed species such as lettuce

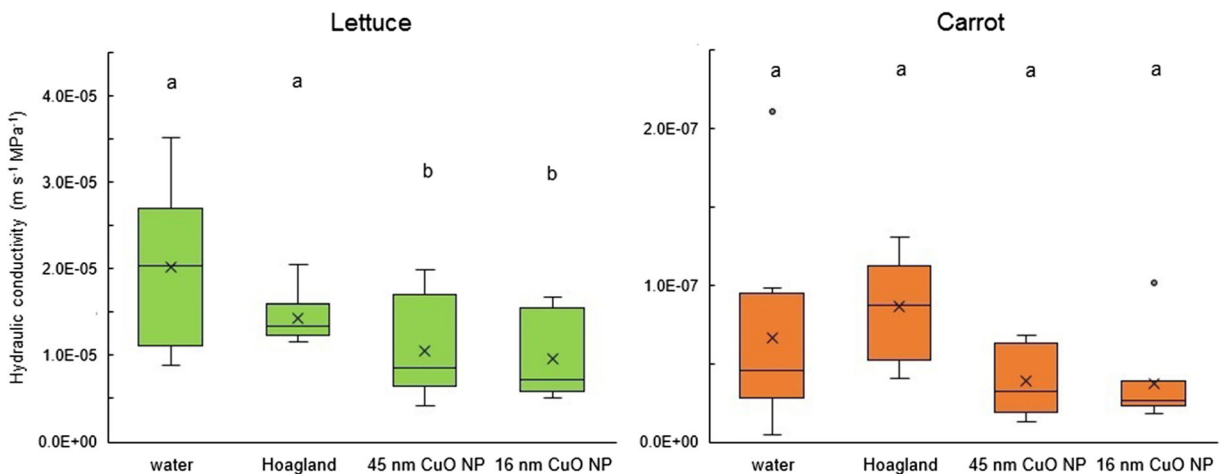


Fig. 5 CuO NP and root hydraulic conductivity. Hydraulic conductivity of lettuce (green) and carrot (orange) plants (21–28 day old) during acute exposure to two types of CuO NPs varying in size (798.9 mg Cu L⁻¹), and de-

ionized water and ¼ Hoagland solution controls. Box plots depict mean (x) and median (solid line). Different letters represent significant differences determined by Tukey's test ($p < 0.05$)

tend to be more sensitive to metal oxide NPs, including CuO (30–50 nm diameter) (Lin and Xing 2007; Tang et al. 2013), due to greater seed surface area to volume ratio (Moon et al. 2014; Shen et al. 2010). Similar seed size of lettuce and carrot may explain similar germination response to CuO NPs and to CuCl₂. Additional factors that may influence germination response to CuO NP exposure include seed coat thickness and composition.

Effect on root morphology

The dose-dependent increase in root diameter induced by CuO NP but not CuCl₂ despite similar Cu²⁺ concentrations indicates an effect of CuO NPs on root growth not solely mediated by Cu²⁺. Root thickening (increased diameter accompanying decreased length) occurred uniquely with increasing CuO NP concentration whereas CuCl₂ only decreased root length. Elongation of nascent roots has been proposed to be a more sensitive measure to nanotoxicity than germination rate because emerging radicles rapidly absorb nutrients and have a high surface area to volume ratio (Wang et al. 2012). Significant effects of CuO NP on root diameter at day 10 but not day 7 suggest cumulative effects that occur more slowly than metal salt controls and may necessitate longer bio-assay periods for detection.

Root thickening can be a physiological response to relieve physical stress on the root apex under conditions of mechanical resistance (e.g., compacted soil)

(Bengough et al. 2006) as well as a response to chemical toxicity of dissolved Cu²⁺ (Adams et al. 2017; Drazkiewicz et al. 2004). Shorter, wider root cells in dense soils (e.g., compact layers, fragipans, high clay content) are thought to be an adaption to enable penetration of dense layers and/or small pores resulting (Bengough et al. 2006; Tracy et al. 2012). Similar cell and root physiological response to CuO NP exposure have been observed to result from hormonally-mediated response to Cu²⁺. For example, decreased cell elongation, increased cell width, and increased abundance of cortical cell columns, occurred for wheat and maize exposed to CuO NP (Adams et al. 2017; Wang et al. 2012, 2016), and for lettuce exposed to rare earth metal oxide NPs (CeO₂, La₂O₃, Gd₂O₃, Yb₂O₃) (Ma et al. 2010). Stress response to reactive-oxygen species (ROS) generated by Cu²⁺ from surface-adhered CuO NP occurred for rockcress (*Aubrieta deltoidea*) and soybean (*Glycine max*) roots, using the same commercially available CuO NP as in the present study (Sigma Aldrich, 45 nm diameter) at similar concentrations (Nair and Chung 2014a, b).

Given the evolutionary demands of soil environments on plant root systems, we propose that the nano-size effect observed for CuO NPs in this and other studies could be triggering a root physiological response developed for clay-rich soils. Root systems of terrestrial plants have evolved in the inherently nanoparticle-rich environment that are soils, because natural NPs exist as clay minerals, metal (hydroxy)oxides, and organic

matter and at concentrations that are orders of magnitude greater than most studies on NPs (Theng and Yuan 2008). For example, allophane is a nano-size mineral (5–50 nm) (Wada 1978) common in volcanic soils in concentrations of up to 26% of soil mass (260,000 mg kg⁻¹) (Parfitt and Wilson 1985), two orders of magnitude greater than the highest NP concentrations used in plant experiments. The ability of plants to grow in soils with high clay content supports the ability of root systems to adapt to NP exposure by the same physiological mechanisms observed in colloid-rich soils (Asli and Neumann 2009). Additional factors may have also influenced observed root thickening in the present study. For example, hydroponic growth conditions likely imparted different physical conditions compared to roots growing in a clay texture soil.

Effects on root function: hydraulic conductivity (K_h)

Compromised water transport during acute exposure to CuO NPs indicates potential negative effects on root functions that could be mediated by mechanical interactions, which may be crop-specific and may occur for NPs larger than generally accepted root pore sizes. Given the short duration of CuO NP exposure (2 h), dissolution of <100 mg L⁻¹ of Cu²⁺ from CuO NP (16 nm diameter) during this period (Fig. 1), and the lack of NP uptake, reduced K_h likely occurred by mechanical effects of CuO NPs exterior to the plant vascular system (root surface or apoplast) (Wang et al. 2016). Blocking or clogging of pores in root cell walls has been proposed as a physical mechanism for observed decreases in K_h for NPs larger than the commonly proposed root pore size maximum of 20 nm (Asli and Neumann 2009). For example, nano-bentonite (1–60 nm) and TiO₂ NP (30 nm diameter) were larger than maize root pores (6.6 nm diameter) yet decreased K_h by up to 25.8% and 33.2%, respectively, during acute exposure (5 h, 1000 mg L⁻¹) (Asli and Neumann 2009). The partial reversibility of K_h reductions was interpreted by the authors as a non-penetrative blocking of apoplastic flow (e.g., shielding of root epidermal cell pores) which could explain similar inhibition of lettuce root K_h by 16 and 45 nm CuO NP in the present study. Reduced K_h could also result from NP obstruction of the diversely sized pores within the apoplast (Jarbeau et al. 1995; Schwab et al. 2016), including those of the cortex cell wall, stele, and even xylem pit membranes, without further translocation into xylem sap. Size-dependent uptake and transport of radiolabeled Fe₃O₄ NPs at rapid timescales (<24 h) has

also been reported in lettuce (Davis et al. 2017). Lettuce seedlings (9 days old) with roots exposed to 9 ± 2 nm and 19 ± 3 nm Fe₃O₄ NPs exhibited similar root NP uptake, but whereas shoot uptake of larger NP stalled within 4 h, continued uptake of smaller NPs over 24 h led to +433% concentration of the smaller NPs in lettuce cotyledons. This indicated a size-dependent bottleneck for movement of 19 but not 9 nm diameter NPs from root to vascular tissues, such as clogging of root cortical cell walls or pit membranes (Davis et al. 2017).

Plant uptake of Cu during acute CuO NP exposure

Our study demonstrates that acute exposure (i.e., high dosage in short time period) can reduce plant root K_h without necessarily entailing CuO NP uptake irrespective of the nearly 3-fold difference in NP size evaluated in this study (16 vs 45 nm). The clogging or surface caking of root pores by NP proposed to reduce K_h (Asli and Neumann 2009) could also decrease the potential for apoplastic entry of NPs. Similar reductions in lettuce root K_h for CuO NP sizes larger and smaller the generally accepted root cell pore sizes of <20 nm diameter (Fleischer et al. 1999) and the absence of Cu species in xylem sap support the hypothesis that CuO NP effects on root water transport can be mediated at the root surface, rather than by symplastic or even apoplastic entry. The absence of detectable CuO NPs or Cu²⁺ in xylem sap during acute exposure is not inconsistent with reported internalization of CuO NP (20–40 nm diameter) by maize roots and transport via xylem because the 15-day old maize plants were grown in CuO NP solution (Wang et al. 2012), an exposure period that engendered cell damage (Miralles et al. 2012; Rico et al. 2011) conducive to internalization of NPs. This process could be a positive feedback because internalized CuO NPs are able to further damage cells and tissues by chemical and mechanical toxicity (Hatami et al. 2016; Schwab et al. 2016). Experimental conditions in our study maximized the possibility of NP internalization because the CuO NPs used were smaller (16 vs 20–40 nm diameter) and at an order of magnitude greater concentrations (1000 vs 100 mg L⁻¹) than CuO NPs that were internalized and transported by xylem in maize (Wang et al. 2012).

Implications for CuO NP in food crop production

These results reveal reduced root function such as water transport during acute exposure of healthy roots not

previously exposed to CuO NPs, indicating that NP-root interactions can challenge root function and thus plant performance independently of internalization of metal oxide NPs and/or dissolved metal ions. Greater likelihood of exposure to NPs of root than shoot systems in both soil and aqueous environments underscores the importance of NP impacts on root K_h . Given the emerging potential of CuO and other Cu-based NP as fungicides in agriculture (Elmer and White 2016; Giannousi et al. 2013; Li et al. 2017; Zhao et al. 2016), this study identifies negative effects of these next-generation NP-based fungicides on root physiology and function of two food crops. Our results are directly relevant to hydroponic food production systems. High concentrations of CuO NPs in this study compared to realistic maximums for irrigation water ($<100 \text{ mg L}^{-1}$) (Servin and White 2016) indicates that on a Cu basis, Cu^{2+} may pose a greater risk than CuO NPs. For scenarios of root exposure to CuO NP in hotspots of fungicide wash-off and accidental spillage during fungicide application or wastewater irrigation expected to entail NP concentrations similar or higher than those used in the highest exposure treatments in the present study (Servin and White 2016), immediate, negative effects on plant root function are possible. Given the greater complexity of soils compared to hydroponic settings and the more likely exposure of food crop root systems in soil environments (Maurer-Jones et al. 2013), further research should examine how NPs compromise root function in soils. Additionally, such assessments should incorporate evaluations of potential physical toxicity on root systems at time-scales relevant to crop production.

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