Cu from dissolution of CuO nanoparticles signals changes in root morphology

CuO NPs form aggregates on the plant root surfaces. NP dissolution, enhanced by factors in the rhizosphere such as organic acids, release Cu, causing root shortening and proliferation of the root hairs close to the root tip. These responses may be due to changes in IAA redistribution from the base of the stele that result in enhanced differentiation of root epidermal cells into elongated root hairs.

Cu from dissolution of CuO nanoparticles signals changes in root morphology

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Key words

Indole acetic acid, Nanoparticles, Nitric oxide, *Pseudomonas*, Root, Wheat

Abstract

Utilization of CuO nanoparticles (NPs) in agriculture, as fertilizers or pesticides, requires understanding of their impact on plant metabolism. Inhibition of root elongation by CuO NPs (>10 mg Cu/kg) occurred in wheat grown in sand. Morphological changes included root hair proliferation and shortening of the zones of division and elongation. The epidermal cells in the compressed root tip were abnormal in shape and file patterning but staining with SYTOX Blue did not reveal a general increase in epidermal cell death. Inhibition of root elongation and proliferation of root hair formation occurred also in response to exogenous indole acetic acid (IAA) supplied through tryptophan metabolism by the root-colonizing bacterium, *Pseudomonas chlororaphis* O6. Altered root morphology caused by the CuO NPs was likely due to release of Cu from dissolution at the root surface because similar changes occurred with Cu ions (≥ 6 mg/kg). Use of a fluorescent probe showed the accumulation of nitric oxide (NO), required for root hair formation, was not changed by the NPs. These findings suggested that dissolution of the NPs in the rhizosphere resulted levels of Cu that modified IAA distribution to causing root shortening but permitted NO cell signaling to promote root hair proliferation.

Keywords

CuO nanoparticles; wheat; root morphology; root hairs; indole acetic acid

Introduction

Novel and varied use of nanoparticles (NPs) extend the potential for their entry into agricultural soils. Indeed NP formulations may be developed to improve nutrient and pesticide delivery to crops. For instance, formulations of CuO and ZnO NPs may provide a long-term and localized source of the essential metals, especially in certain soils where Cu and Zn have limited bioavailability (Servin et al. 2015). In addition to purposeful application of NPs, unintended contamination of agricultural soils with NPs may occur through accidents, water recycling, or use of sludges bearing NPs from downstream waste facilities (Keller et al. 2013). This paper examined plant responses to CuO NPs, which are part of the array of diverse Cu-containing nanoparticles already in commercial use (Rubilar et al. 2013). Environmental factors which alter dissolution of the metal-containing NPs and their bioavailability play important roles in determining toxicity, especially for the metals such as Cu that are both essential for cellular function yet exhibit very sharp toxic thresholds (Gajjar et al. 2009).

Many laboratory studies demonstrate dose-dependent phytotoxicity of CuO NPs. Root shortening occurs in a range of plants when grown in hydroponics, sand, and soil conditions after exposure to CuO NPs (Wang et al. 2012,; Dimkpa et al. 2013; Shi et al. 2014; Peng et al. 2015; Ebbs et al. 2015; Nair and Chung 2015). Transport of intact CuO NPs into maize roots and their redistribution *in planta* via xylem and subsequently phloem is documented (Wang et al. 2013). However, some NP dissolution occurs with CuO NPs in the growth matrix based on detection of copper sulfide deposits in leaf tissue of wheat seedlings grown with CuO NPs (Dimkpa et al. 2013). Similar findings for rice seedlings of uptake of CuO NPs and its transformation into other Cu complexes are reported (Peng et al. 2015).

This paper focuses on the changes in morphology occurring in wheat roots imposed during growth with CuO NPs, conditions which would allow responses both to NPs, and to Cu released from the NPs. Released Cu could be as free ions or as Cu-ligands formed by association with compounds in the rhizosphere of diverse origin (Misra et al. 2012). Compounds such as soil humic acids (Peng et al. 2015; Grillo et al. 2015), or those produced by the soil biota, or plant metabolites in root exudates could

influence metal release and the biological response (Martineau et al. 2014;Calder et al. 2012).

Cu is one of the essential metals for all cells, being involved in normal metabolic functions (Festa and Thiele 2011), However, there is a sharp toxicity threshold because at higher doses toxic effects of ion exposure are manifest with such responses as root shortening (Kopittke and Menzies 2006; Michaud et al. 2008; Pena et al. 2015). The reduction in elongation in *Arabidopsis* roots exposed to Cu ions correlates with the abnormal distribution of auxin, indole acetic acid (IAA) (Yuan et al. 2013, Wang et al. 2015). IAA is a major player in root development (Overoode et al. 2010). Regulation of IAA levels within the different cell files of the root is involved in many root functions including growth, gravitropism, lateral root elongation and root hair formation (Overoode et al. 2010, Peer et al. 2011). Various pathways for IAA synthesis exist in plants with tryptophan being the common precursor (Mano and Nemoto 2012). Most of the IAA is produced by the shoots and is delivered to the root tips where redistribution from the central stele to the other cells occurs in an “inverted fountain effect” (Overoode et al. 2010). The flow of IAA involves discrete influx and efflux pumps in the cell membranes that allow this plant growth regulator to enter, accumulate within the cell and exit the cells only in a defined direction Jones et al. (2009). The efflux pumps include those formed with PINFORMED (PIN) proteins which have defined cellular locations within different lineages of the plant organs (Krecek et al. 2009). The protein PIN1, and an ABC - efflux protein, are associated with the polar flow of IAA from the shoot to the root tip, while PIN4 loads IAA into root cap cells prior to shootward movement through epidermal and cortical cells (Peer et al. 2011). Reduced expression from PIN1 occurs upon exposure of *Arabidopsis* roots to Cu ions with consequent reduction of IAA in the columella and lateral root tip cells (Yuan et al. 2013). IAA redistribution results in accumulation in the root apex and correlates with reduced primary root elongation (Yuan et al. 2013). PIN function is also connected to root hair elongation in *Arabidopsis* because higher IAA levels increase root hair length (Ganguly et al. 2010). Impaired cell division is shown for wheat exposed to Cu ions so that cortical cells have longer lengths (Pena et al. 2015) and the distance between root tip and root hair formation is reduced (Kolbert et al. 2012).

Exogenous IAA also can cause changes in root morphology. Previous work with *Azospirillum* isolates links IAA production by these bacteria to growth of wheat seedlings with short roots and proliferation of root hairs (Dobbelaere et al. 1999). Mutants of *A. brasilense* that lacked IAA production no longer cause these changes in wheat, whereas addition of exogenous authentic IAA caused root shortening and increased root hair formation (Dobbelaere et al. 1999). Exogenous IAA results in accumulation within the columella and stele, as is observed with IAA synthesized *in planta* (Shibasak et al. 2009).

Nitric oxide (NO) is another signaling factor that regulates root growth and development interactively with other growth regulators (Stohr and Stremlau 2006). The complex interaction between IAA and NO varies with root cell location as demonstrated from studies of Cu ion repression of *Arabidopsis* root elongation (Kobert et al. 2012). Studies suggest that NO decreases cell division and promotes cell differentiation through reduced activity of PIN1 (Fernandez-Marcos et al. 2011), a transporter for IAA flux in *Arabidopsis* (Yuan et al. 2013).

The potential for CuO and Cu NPs introduced into the rhizosphere to serve as point sources for Cu release and modulation of key signaling molecules in root development motivates investigation of this class of NPs in agriculture. In the present studies microscopy was used to examine how exposure to CuO NPs alters behavior of cells in the root cap, the meristem, and the zone of differentiation of root epidermal cells to root hairs. The wheat seedlings studied were raised in sand, chosen for the absence of potential soil metal chelators, with and without addition of CuO NPs. Growth with Cu ions was also studied to understand the potential importance of NP dissolution. In *Arabidopsis* and lettuce, IAA is involved in determining which epidermal cells develop into root hairs and in regulating root hair elongation (Lee and Cho 2013). Consequently, the distance between root tip and the zone of differentiation, as well as root hair density and length, was examined to provide visual indications of Cu-induced IAA modulation. The meristematic region was imaged to determine effects on cell files and shape. To probe the role of IAA we generated exogenous IAA at the root surface through the metabolism of tryptophan by the root-colonizing bacterium, *Pseudomonas chlororaphis*

isolate O6 (*PcO6*). In *PcO6* the conversion of tryptophan to IAA increases slightly in the presence of CuO NPs or Cu ions, unlike the inhibition observed with ZnO NPs (Dimkpa et al. 2012). Microscopy was used to determine the effect of increased exogenous IAA on root tip morphology to those induced by CuO NPs. Cell death was compared between exposed- and nontreated wheat seedlings using SYTOX Blue, which penetrates only cells with damaged plasma membranes and results in fluorescence as it associates with DNA (Truernit and Haseloff 2008). For instance CuO NP-induced cell death in germinated rice seedlings in the meristematic and elongation zone is proposed to be related to stunted growth (Peng et al. 2015). The accumulation of NO in the wheat root tips of this study was determined by using a fluorescent indicator, DAF-FM DA, able to detect 5 nM NO (Foissner et al. 2000).

Materials and methods

Plant growth

Wheat seedlings, cultivar Doloris, were grown from surface-sterilized seeds in sterilized, wetted sand following gnotobiotic procedures as described (Watson et al. 2015). Characterization of the CuO NPs (size, composition, and dissolution rates) is found (Dimkpa et al. 2012, 2013). The CuO NPs were added, at 3, 10, 30, and 300 mg Cu/kg sand according to the study, prior to sterilization. Other microcosms contained Cu ions from CuSO₄ at defined concentrations, 2, 10, 15 and 20 mg Cu ion/L (i.e. 0.6, 3.6, 4.5 and 6 mg/kg sand) according to the study. Five replicates were run for each treatment. Each microcosm contained 5 seedlings. Studies were replicated a minimum of three times. Seedlings were removed at 7-d growth and root and shoot lengths were measured.

Border cell release

Excised root tips were immersed in 100 µl sterile water for 30 min before staining with Toluidine blue to determine release of border-like cells by light microscopy using a Zeiss Standard microscope with 40 x objective. Any cells that were partially or completely released were scored positive for release. Fifteen plant root tips were examined for each of three replicated studies.

Confocal microscopy analysis

Imaging was performed with 7-d seedlings grown with and without treatments of CuO NPs or Cu ions. The plants were carefully lifted from the sand and the roots washed in sterile distilled deionized water (DDW). Tip sections (5 mm) were excised and either examined without staining or processed with dye solutions, as described in the sections below. The epidermis of unstained live root tips was viewed in transmission mode with 458 nm light using a Zeiss LSM 710 with 63 x objective. Images were taken with successive fields of view overlapping 10 -20 %. Images were processed uniformly with Adobe Photoshop.

To visualize surface root cells with defective plasma membranes, the dye, SYTOX Blue (Life Technologies/Thermo Fisher Scientific Carlsbad CA), was used; this dye is reported only to stain cells with damaged plasma membranes permitting the dye to interact with DNA in the nuclei and to emit fluorescence when excited. The stock dye was diluted to 500 μ M according to manufacturer's instructions before adding to the washed root tips at a final concentration of 1 μ M. After 15 minutes the tips were mounted between a glass slide and coverslip and fluorescence was observed with the LSM 710 with excitation at 458 nm and emission at 463-509 nm. Autofluorescence of the root tips was examined using tissues not treated with SYTOX Blue. For each study at least three root tips were examined for one treatment and at least three independent studies were performed before representative images were selected. The images were uniformly processed for brightness and contrast.

To determine accumulation of NO in the plant cells, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) (Life Technologies, Carlsbad CA) was used; this dye has been used previously to characterize NO accumulation in plant cells (Foissner et al. 2000). The chemical diffuses through the plant cell membrane and deacetylation by cellular esterases produces the DAF-FM molecule which on interaction with NO becomes fluorescent. Root tips, prepared as described above, were immersed in 5 μ M dye from a stock of the compound in dimethyl sulfoxide (DMSO) (Life Technologies). Use of a range of dye concentrations demonstrated that this concentration was appropriate with the plant tissues. After 30 min incubation with the

dye, root tips were transferred into sterile DDW for 15 min. Fluorescence was examined with 488 nm excitation and 493-566 nm emission. Autofluorescence of the root tips was examined using tissues without treatment with DAF-FM-DA. For each study at least three root tips were examined for one treatment and at least three independent studies were performed before representative images were selected. The images were processed uniformly to enhance brightness and contrast.

Scanning electron microscopy (SEM)

SEM was performed with a FEI Quanta FEG 650 equipped with an Oxford X-Max EDS housed in the Microscopy Core Facility at Utah State University. All plants were grown for 7 d and the roots were harvested carefully from the sand matrix and washed by immersion in sterile water. Fresh root tissues from 7 d plants were used for images obtained from the root caps within 2 h of harvest. Dried roots were used for SEM/EDS analyses. For each treatment, at least three roots grown with or without 300 mg Cu/kg from CuO NPs were examined. With EDS analysis, element distribution was determined at more than one location across a root surface. For point EDS analysis, two to three particles were selected for each root.

Growth with exogenous, biologically-produced IAA

Surface-sterilized seeds were inoculated with *PcO6* (10^6 cells/ml) by suspension for 10 min. After draining onto sterile filter paper, seeds were directly transferred into the sand matrix containing 1 g/L tryptophan (50 ml/300 g sand). The effect of tryptophan alone was studied by planting boxes with noninoculated seeds. Colonization by *PcO6* was verified by transfer of 7 d-old roots into 10 ml sterile water. Bacterial cells from the root surface were physically released by vortexing for 1 min. Serial dilutions of the suspensions were plated onto rich Luria broth medium. *PcO6* grew as bright orange colonies and the colony forming units (cfu)/g root tissues were determined from colony

growth after 48 h. The roots of plants grown with and without *PcO6* colonization were examined by microscopy as described above.

Results

Inhibition of root elongation by CuO NPs

Exposure of wheat seedlings to CuO NPs, continuously from the time of planting the seeds in sand containing NPs to harvest, caused reduction in root growth with a trend towards greater effects with increasing dose as shown in Figure 1. No reduction in root growth was observed with 3 mg Cu from CuO NPs /kg sand (data not shown). Statistically significant inhibition of root elongation was observed with doses of 10, 30, and 300 mg Cu/kg sand from the NPs compared with the control. No statistically significant reduction in shoot length was observed with these doses of 3 to 300 mg Cu/kg sand from NPs (Fig 1). Reduction in root elongation was duplicated by amendment of sand with Cu ions at 4.5 and 6 mg/kg sand; root growth was not affected by 0.6 and 3.6 mg Cu ion/kg sand doses (Fig 1). Reduced shoot growth occurred with the 4.5 and 6 mg Cu ion/L doses.

Contact between wheat root surfaces and CuO NPs

Imaging of the epidermis of root tips of plants grown with CuO NPs revealed aggregates of CuO NPs both on the root surface and the root hairs (Fig. 2). Such aggregates were not present on roots observed from plants grown without exposure to CuO NPs (Fig.2). SEM EDS analysis of root surfaces from plants grown with CuO NPs confirmed the presence of Cu in adherent particles. Signals from Cu were absent from root surfaces of plants grown without exposure to Cu (Supplemental Figure 1).

Effects of CuO NPs and Cu ions on root morphology

Root cap and border-like cells: Light microscopy analysis of the wheat root tips revealed the presence of cells typical of border cells at the root cap. When stained with toluidine blue, release of brick-shaped cells from the tip was observed by light microscopy over a 30 min period. No difference was observed between the average cell release from root tips of seedlings grown with or without CuO NPs; however, the extent of release from

different root tips was highly variable and, thus, could have masked any difference (Supplemental Table 1). Examination of root caps at higher magnification by SEM under environmental mode did not reveal changes in root morphology between growth with and without CuO NPs (Supplemental Figure 2).

Meristem regions: Microscopy of the surface of nonstained living root tips revealed elongated columella cells adjacent to smaller irregularly sized cells in the region of the quiescent center-meristem for seedling grown without Cu challenge (Fig 3-A- 1). The root surface at the meristematic region of control wheat seedlings showed aligned files of regularly shaped cells (Fig 3 A-1 to A-3). Aligned files are also visible with SEM (Supplemental Figure 1). However, the surface cells from the root tips of seedlings grown with CuO NPs were irregular in shape and alignment (Fig 3 B-1 to B-3). The walls between cells were wavy rather than straight. The intracellular structure of the cells grown with CuO NPs also was highly granulated. CuO NPs were observed associated with the epidermal cells of the wheat seedling roots throughout this region. Highly enlarged cells, granulated cells and cells with poor alignment also were observed at the surface of the meristem region for root tips of the root-defective plants grown with 4.5 and 6 mg Cu ions/kg (Figure 3 C and D). At lower Cu ion doses (0.6 mg/kg Cu ions) when there was no impact on root elongation, the cells were regularly shaped and in aligned files in the meristem area (data not shown) resembling those of roots grown without Cu exposure (Figure 3 A).

Zone of differentiation: the root hairs: The zone of differentiation, where the root epidermal cells differentiated into root hairs, was closer to the root tip for plants grown with CuO NPs treatment or with Cu ions than for the control plants (Fig. 4 A). The ion doses, which resulted in significant reduction of root elongation (Fig. 1), showed the zone of differentiation at similar distances from the root tip to those that were observed with the CuO NPs treatments (Fig. 4 A, B). Additionally the root hairs in the zone where differentiation first occurs were considerably longer for the CuO NP-exposed roots than those seen in the equivalent zone for the control plants (Fig. 4 C). In some images with CuO NPs treatments curling back of root cap cells was notable. Growth with Cu ions also caused root hairs to be longer but with a greater variability than observed with

growth with the NPs as can be observed by the large error bars in these data sets (Fig. 4 C).

Proliferation of root hair formation and root shortening by exogenous IAA

The involvement of IAA in causing root shortening and root hair proliferation in wheat seedlings under our growth conditions, was supported by the results of microscopy of wheat roots colonized by *P. chlororaphis* isolate O6 (*PcO6*) when tryptophan was added to the growth boxes (Supplemental Figure 3). These data confirmed studies with wheat with additions of authentic IAA or growth with another root-colonizing microbe (Dobbelaere et al. 1999). Growth with *PcO6* in the presence of tryptophan, caused inhibition of root elongation ($60 \pm 5 \%$) and proliferation of root hairs close to the root tip compared with growth with *PcO6* alone (Supplemental Figure 3 A, B). Colonization of the roots by *PcO6* in the absence of tryptophan did not alter the distance to root hair production from the root tip compared to noninoculated roots (Supplemental Figure 3A) and imaging along the root surface (Fig. 3 E) showed that the cells in the root tips were in linear files and not misshapen as were the roots from plants raised with Cu exposure.

Cell death

Root tips were stained with SYTOX Blue to reveal dead cells on the root surface in the effort to determine the extent to which CuO NP-stress induced cell death. Confocal images of the root tips of control plants showed the presence of dead cells arranged in streaks after the root cap in the roots grown without Cu challenge (Supplemental Fig 4). Cell death also was apparent in the root cap cells. The files of dead cells along the root did not occur on seedlings raised in vermiculite suggesting that some of these dead cells arose due to physical damage caused by sand grains during growth (Supplemental Figure 4). Sharp points and rough edges on the sand grains were visible and their surfaces possessed aggregates of CuO NPs when this was added to the growth matrix (Supplemental Figure 4 B). The finding of aggregates of CuO NPs coupled with the observation that particles of sand adhere to the plant roots upon harvest indicated that the root epidermal cells also would contact the NPs on the sand grains as well as those in the sand pore waters. Studies of NP effects in vermiculite were not conducted

because of the problem of strong sorption of the particles and metal ions onto the vermiculite particles. The extent of cell death in the root cap and the region of division and elongation did not appear to differ between the roots of control and Cu-exposed seedlings grown in sand (Fig. 5). However, imaging of the root hair zones showed a greater intensity of SYTOX Blue staining in the initial root hair zones for plants grown with CuO NPs exposure and to an even greater extent treatments with Cu ions compared to the controls (Fig. 5). There was no autofluorescence of these tissues under the imaging conditions (Supplemental Figure 5 A); pretreatment of the root tip with tertiary butoxide resulted in intense blue fluorescence of the cells indicating extensive death of root surface cells from oxidative damage (Supplemental Figure 5 C).

NO generation in root tips

Root hair formation and their elongation are associated with NO signaling (Sanz et al. 2015). The dye DAF-FM DA within the cells cytoplasm becomes deacetylated and fluorescent after reacting with NO; the dye is nonreactive with reactive oxygen species. There was no autofluorescence of the root cells under these conditions (Supplemental Figure 5 B). Figure 6 A shows fluorescence from DAF-FM DA in the root cap and meristem cells. In Figure 6 B images for the root hair zones are shown. Similar patchworks of cells with and without fluorescence were observed for roots grown with and without CuO NPs or Cu ions. Images for the differentiated zone of the roots grown with CuO NPs /or Cu ions (Fig. 6 B) were less clear than for the control roots because the overlay of extensive numbers of root hairs above the epidermal surface physically obscured fluorescence from the root surface cells.

Discussion

Growth of wheat roots in sand containing CuO NPs changed root morphology, with elongation being inhibited at doses above a threshold concentration, about 10 mg Cu/kg

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4 338 sand from CuO NPs. The wheat root tips were surrounded by large brick-shaped cells
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6 339 and a mucilaginous layer both typical of border cells studied in other plants (Miyasaka
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8 340 and Hawes 2001). Release of border cells and the production of mucilage are
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10 341 suggested to be a protective response against metal ion challenges (Miyasaka and
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12 342 Hawes 2001). The association of metal ions with dead border cells is proposed to
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14 343 contribute to metal tolerance (Tamas et al. 2005). In our studies, with the seedlings
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16 344 grown in the solid matrix sand, there was no increased border cell death or border cell
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18 345 release or root cap morphology when seedlings were grown with Cu exposure from
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20 346 NPs. Thus, the border cells in the root cap have a demonstrated protective effect for
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22 347 roots exposed to CuO NPs. However, dramatic changes in the meristem zone of the
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24 348 roots, such as increased cell size, misshapen cell margins, and loss of alignment into
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26 349 regular cell files, demonstrated effects on division and elongation from growth with
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28 350 exposure to either CuO NPs or Cu ions. The fact that similar changes were observed in
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30 351 our studies with wheat roots for Cu ions points to a role for Cu released from CuO NPs
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32 352 dissolution in the plant responses. Thus, we stress the importance of NPs acting as
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34 353 localized sources for metal release in the plants' responses to the NPs. Aggregates of
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36 354 CuO NPs were abundant on the root hairs as well as the surface cells for the plants
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38 355 grown with CuO NPs. The released Cu could account for the changed meristematic
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40 356 activity resulting in the dramatic shortening of the distance between the root tip and the
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42 357 zone of differentiation of epidermal cells into root hairs. The growth of plants in the solid
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44 358 sand matrix as opposed to hydroponic conditions permitted the effects on root hair
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46 359 development to be visualized. Root hair development may not occur to the same extent
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48 360 during hydroponic plant growth used in other root nanoparticle studies.

47 361 Our work does not support that death of epidermal root cells was a major
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49 362 contributor of the morphological changes induced by the CuO NPs, as used at doses up
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51 363 to 300 mg Cu/kg sand. Increased apical meristem cell death due to Cu ions has been
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53 364 reported for *Arabidopsis* (Lequeux et al. 2010). It is possible that treatment with the
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55 365 SYTOX Blue did not access cells below the epidermis to reveal their cell death.
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57 366 Additionally the examination of the surface cells for cell death induced by CuO NPs may
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59 367 be complicated by physical damage to such cells during growth through the sand
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61 368 grains. The dead or damaged cells could possibly promote NP entry into the root and
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increase their bioactivity. Sand particles in field soils also could cause similar physical damage to the surface of the growing root. Reduced root elongation was observed when wheat was grown in a sandy (85 % sand), acidic field soil amended with CuO NPs (Anderson et al. 2016).

The inhibition of root elongation with CuO NPs resembled findings for work with Cu ions in *Arabidopsis* where inhibition of elongation by Cu ions was correlated changes in IAA levels due to altered transport (Yuan et al. 2013). The mechanism for the Cu ion-induced block in *Arabidopsis* involved reduced expression from the gene encoding PIN1 responsible for Cu efflux from the most basal central cylinder cells prior to shoot-directed redistribution (Blilou et al. 2005; Yuan et al. 2013). There was no effect on the expression of genes encoding another efflux pump PIN2 or an IAA-influx gene in the *Arabidopsis* roots treated with Cu ions (Yuan et al. 2013) but other studies suggest Cu ions induced change in additional genes associated with IAA homeostasis in *Arabidopsis* roots (Peer et al. 2011). It is possible that cell injury caused by growth through the sand growth matrix also enhanced IAA responses in the root cells. Such wounding could lead to the synthesis of wound hormones, such as jasmonic acid (JA) and ethylene. These regulators could have an impact on IAA in the plants because JA is reported to stimulate IAA synthesis and upregulate genes controlled by IAA and ethylene (Lee et al. 2013).

The observed changes in wheat root morphology caused by growth with Cu could be correlated with impaired IAA efflux at the base of the stele, altering root division and elongation if the mechanisms of Cu-toxicity in *Arabidopsis* also occur in wheat. Imaging of the wheat root meristematic regions confirmed that the epidermal cells were misshapen and lacked regular cell file organization with exposure to CuO NPs or Cu ions. Dramatic changes also were observed at the zone of differentiation that could involve changes in IAA levels. IAA is key to regulating the process of differentiation of root epidermal cells to root hair cells, as well as the elongation of root hairs (Jones et al. 2009; Ganguly et al. 2010; Lee et al. 2013). Change in IAA flux in root hair cells has been implicated as a regulatory factor for formation and development of these differentiated cells (Ganguly et al. 2010). Whether there is discrete inhibition of

IAA efflux/influx pumps associated with root hair cells awaits investigation in wheat. The use of exogenous IAA, produced biotically by a root-colonizing pseudomonad, adds additional evidence that changes in level of the IAA triggered abnormal root morphology. However irregular cell files observed at the root surface in the Cu-exposed seedlings were not seen with the shortened roots arising from IAA treatment suggesting that cell deformation was a Cu response.

The use of a fluorescent indicator, DAF-FM DA, to detect cellular accumulations of NO showed that growth with Cu from the CuO NPs or ions did not impair NO formation. In *Arabidopsis* roots Cu ion treatment resulted in increased NO accumulation (Kolbert et al. 2012) through IAA-stimulation of NO synthesis, largely from nitrate by a nitrate reductase (Kolbert et al. 2008). In the wheat roots NO accumulation was observed in epidermal cells developing into root hairs and within the long mature root hair cells, whether or not the seedlings were grown with CuO NPs. Root hairs are associated with uptake of water and nutrients into the root and their abundance on the root tips of plants grown with CuO NPs may have allowed shoot elongation to continue at near control levels through growth only to the early seedling stage of our studies.

In summary the changes in root morphology caused by growth with CuO NPs can be attributed to the localized release of Cu from contact between the root surface cells and the NPs. Root cell functions, such as secretion of protons and factors to chelate released metal ions, may be important in conditioning the extent of dissolution of the CuO NPs in the rhizosphere. The ability of CuO NPs to modify root structure is an important factor to be considered in the formulation of such NPs in products intended for soil application for plant nutrition or as antimicrobial agents. It is possible that the proliferation of the roots hairs induced by the CuO NPs may be a property that is advantageous under certain conditions such as drought and harvesting of scarce nutrients.

Supplementary data

Supplemental Table 1 Release of border cells from wheat root caps from control plants and plants grown with two concentrations of CuO NPs.

Supplemental Figure 1. SEM /EDS imaging of Cu for root tips of plants grown with and without CuO NPs.

Supplemental Figure 2. Environmental SEM imaging of root caps for root tips of plants growth with and without CuO NPs.

Supplemental Figure 3A and B. A. Colonization of roots with *PcO6*. B. Growth of wheat seedlings with *PcO6* but with and without added tryptophan.

Supplemental Figure 4 A and B. A Effect of growth matrix on death of root surface cells. B. Imaging of sand grains with and without incubation with CuO NPs and Cu ions.

Supplemental Figure 5. A and B. Images of controls for autofluorescence. C. Images of positive staining for dead root tip cells.

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Fig 1.

Effect of Cu challenge from CuO NPs or Cu ions at designated concentrations on plant growth.

Root and shoot lengths were measured for each seedling. Data shown are averages of three independent studies each with 5 replicates for each treatment with 5 seedlings in each replicate. The mean and standard errors are provided. The letters above the bars indicate significant differences for either shoot height or root length at P=0.05 for each treatment when analyzed separately.

Figure 2.

Presence of aggregates of CuO NPs on roots of plants grown for 7 d in sand amended with 30 mg Cu and 300 mg/kg from CuO NPs. Aggregates are not apparent from roots grown without the NPs. Images taken with the LSM 710 are representative of at least 10 different root samples. Bar is 200 μ m. The arrows show aggregates of NPs on the root hairs and cell surface.

Figure 3 Images of root tip surface cells at the meristem regions of wheat seedling root tips after growth without (A 1,2,3,) or with 300 mg Cu/kg from CuO NPs (B 1,2,3) or with Cu ions at 4.5 mg/kg (C 1,2,3) and 6 mg/kg (D 1,2,3) or with tryptophan and colonization by *P. chlororaphis* O6 (E 1,2,3). The bars are 50 μ m for each image. Images labeled as 1 show the junction between the elongated columella cells and the predicted position of the quiescent center (QC). Images 2 and 3 are progressively further towards the zone of differentiation along the root. In B, NPs on the root surface are also noted. Data are typical of roots of 5 different plants imaged by confocal analysis with no staining procedures or fixation. Bar is 50 μ m.

Figure 4 ABC. Growth with CuO NPs or Cu ions changes root morphology: distance between tip and root hair formation is reduced. All data are from 7 d wheat root tips grown in sand with and without Cu from CuO NPs or Cu ions. The distance was measured from a minimum 20 different hairs from 10 roots. A: Images of root tips from plants grown with CuO NPs, Cu ions or without challenge. B: The relationship between distance to the initial formation of root hairs from the root tip in plants grown with CuO NPs and Cu ions from CuSO₄. C: The relationship between length of initial root hairs and Cu treatments.

A. Comparison of root tips from seedlings grown without treatment or with CuO NPs or Cu ions. Images are typical of three different roots from three independent studies all grown for 7 d (n=9). Shown are root tips grown: as a control with no Cu additions, with 3 mg Cu from CuO NPs /kg sand, with 30 mg Cu from CuO NPs /kg sand, with 300 mg Cu from CuO NPs /kg sand (note the loss of root cap integrity with this treatment), with 4.5 and 6 mg Cu ions/kg sand.

B. Distance between root tip and root hair initiation.

C. Root hair length

Figure 5 Images of root tips treated with SYTOX Blue to reveal dead cells on the root epidermis.

Images are from root tips treated with SYTOX blue for plants grown with no treatment, CuO NPs and Cu ions. Bar is 200 μ m. These are representative images from three separate studies each performed with three roots for each treatment.

Figure 6 Fluorescence in wheat root tip cells indicative of NO accumulation as revealed by fluorescence after exposure to the dye, DAF-FM DA. Wheat seedlings were grown for 7 d in sand without and with (A) 300 mg/kg CuO NPs or (B) 6 mg/kg Cu ions. Excitation was at 495 nm and emission was recorded at 515 nm. Images are typical of three different studies where three root tips were examined. Bar is 200 μ m.