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Soil-derived fulvic acid and root exudates, modified by soil bacteria, alter CuO nanoparticle-induced root stunting of wheat *via* Cu complexation†

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CuO nanoparticles (NPs) are explored as fungicides and fertilizers, and are increasingly likely to be applied to agricultural soils. Consequently, interactions of CuO NPs with soil pore water (SPW) components, plants, and microbes must be understood. These experiments examined whether dissolved natural organic matter (DNOM) from SPW, or root/bacterial exudates, changed wheat (*Triticum aestivum* L. v. Deloris) responses to 100 mg kg^{-1} (Cu/sand) as CuO NPs. Seedlings were grown in sand with 3.34 mM $Ca(NO_3)_2$ or one of three SPWs, differing in DNOM concentration and composition. At 10 days post-germination, CuO NPs stunted roots by 59% in the 3.34 mM $Ca(NO_3)_2$ and 26–35% in the three SPWs compared to plants grown without NPs. Malate, citrate, gluconate, and 2′-deoxymugineic acid (DMA), were elevated 1.3 to 5-fold in the rhizosphere with CuO NPs present. Cu was bioavailable through metallo-organic complexes, including Cu–DMA and Cu–gluconate. Fulvic acid in SPWs mitigated CuO NP-induced wheat root shortening. *Pseudomonas chlororaphis* O6 eliminated malate and citrate in the rhizospheres, reduced rhizosphere dissolved Cu ~18–66%, and reduced root Cu 39% across all SPWs while enhancing root stunting ~17% more across all SPWs than non-inoculated wheat grown with CuO NPs. Thus, both SPW components and root microbial colonization influenced wheat responses to CuO NPs. These interactions are likely in agricultural soils with additional processes, such as ion sorption, to influence CuO NP phytotoxicity, highlighting the importance of considering not just the target plant, but soil properties and associated microbiomes when evaluating impacts of NPs in agricultural usage.

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Environmental significance

Nano-enabled agricultural products, including Cu and Cu oxides, are already on the market, but the chemistry and effects of NPs in the wide variety of worldwide agricultural soils is under-characterized. Application of nano-enabled agricultural products may result in unintended consequences to target and non-target organisms. The dissolution of CuO NPs in the rhizosphere results in metallo-organic complexes that have a range of low to high bioavailabilities to wheat. Most metallic NPs may likewise dissolve in the rhizosphere, form metallo-organic complexes, and enter crops. This effect may be beneficial, such as augmentation of cereal grains with micronutrients for crop yield and human nutrition, or harmful, such as stunting of crop growth, dependent on soil chemistry factors and NP dose.

Introduction

Nanoparticles (NPs, particles less than 100 nm in at least one dimension) have multiple potential uses in agriculture.[1] CuO NPs in particular may be included in formulations for fungicides,[2] fertilizers,[3,4] and treatments to alleviate drought stress.[5–7] CuO NPs may also partition into biosolids generated in wastewater treatment plants that may be applied to field soil.[8–10] CuO NPs in these applications will inevitably contact soils through overspray, leaf litter of foliar applications, or intentional application to soils or seeds. Thus, the fate, transformations, and interactions of CuO NPs with soil, microbes, and plants must be further studied to more safely and effectively inform NP applications in agriculture.

CuO NPs display dose-dependent toxicity for soil microbes and crops, as shown by studies with wheat and a pseudomonad in well-defined media.[11–13] However, native

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soils contain a rich mixture of diverse microbes, dissolved natural organic matter (DNOM), and dissolved minerals and gases, among other components in the soil pore waters (SPWs). Studies of CuO NPs and wheat or microorganisms in soils underscore that complex mechanisms and (bio) chemical reactions in the soil environment affect toxicity and plant response to CuO NPs. For example, 500 mg kg^{-1} CuO NPs aged in soil for 28 days at pH 6.5, but not fresh dosing of the CuO NPs, reduced root length of 14 d old wheat.[14] The authors attribute the toxicity of the aged CuO NPs to the increased dissolution present at the beginning of the test for the aged NPs. Dissolution is also influenced by pH[1,14–17] and root exudates,[1,14,18] with spatial gradients existing outward from the rhizosphere.[14] Gao *et al.*[17] reported that different chemical composition of DNOM in soil will complex Cu to varying extents, thus influencing solubility of CuO NPs.

Interactions of DNOM or pH with the CuO NPs may change how the NPs affect plants. Higher pH thermodynamically limits CuO solubility, which is least soluble between 9–11.[19] Complexation reactions between dissolved Cu and inorganic or organic ligands in the soil solution may however drive the dissolution of CuO, altering Cu uptake into wheat plants.[20,21] Even in calcareous soils, sufficient DNOM may exist to complex and dissolve Cu. Furthermore, wheat root exudates include the organic acids malate and citrate, as well as the phytosiderophore 2′-deoxymugineic acid (DMA)[13] which will alter plant–Cu interactions. Growth of wheat with CuO NPs in wetted sand, with a background electrolyte of $Ca(NO_3)_2$, increases the exudation of these Cu-complexing metabolites.[13] Also present in SPWs as part of the DNOM are the humic and fulvic acids that can bind Cu[22] and coat CuO NPs.[23,24] DNOM increases dissolution of Cu from CuO NPs by complexing with released metal; however, the role of these SPW complexing compounds in rhizosphere interactions between CuO NPs and plants is mostly uncharacterized. Understanding these interdependent processes would be valuable for purposeful agricultural soil amendment with CuO NPs as fertilizers or pesticides.

In order to investigate the mechanisms involved in the effects of CuO NPs on plants and alleviation of symptoms, geochemical and mechanistic toxicity models are useful to identify the active soil factors. Free Cu^{2+} ions are the cause of toxicity under the biotic ligand model, a commonly used toxicity model that successfully accounts for Cu toxicity in wheat roots.[21,25,26] Complexation of Cu,[21] and competition with cations Ca^{2+}/Mg^{2+} for root sorption sites[21,25,26] limit the bioavailability and, thus, influence the toxicity of free Cu^{2+} ion. Therefore, under the biotic ligand model, roots should grow longest at high pH, DNOM, Ca^{2+}, and/or Mg^{2+} for equal concentrations of dissolved Cu. Geochemical modeling of rhizosphere components will allow the distribution and speciation of dissolved Cu in the solution phase to be predicted and these values used to examine the fit with the predictions of the biotic ligand model.

The impact of plant-associated and soil bacteria on the overall system also is not often considered in the rhizosphere processes that govern plant responses to NPs and thus needs to be considered to construct a more accurate and predictive model.[14] Microbial colonization changes wheat root morphology[27] and moderates the influence of abiotic and biotic stressors, such as drought[7] or pathogens,[28] in the host plant. Microbes metabolize root exudates, and secrete their own compounds that could interact with the NPs, again through coating and modifying dissolution,[29] or interact with the root, altering stress resistance mechanisms and/or root exudate products. Pseudomonads, common soil bacteria, particularly exude pyoverdines[30] which are siderophores but may interact with Cu, similar to how the phytosiderophore DMA interacts with Cu. In turn, these processes may affect NP phytotoxicity.

Given the complex and unknown interactions between plant roots and their exudates, root-colonizing bacteria and their metabolites, DNOM present in soils, and inorganic NPs, the subject should be further studied. The objectives of this study were therefore to examine the effects of differing SPW chemistries, a root-colonizing bacterium, and their interaction, on the phytotoxicity and uptake of the CuO NPs in wheat.

Materials and methods

Experimental design

The experiments were designed to test the effects of DNOM from three SPWs, and the metabolites in wheat root and *Pseudomonas chlororaphis* O6 (*Pc*O6) exudates on wheat grown in the presence of CuO NPs. The SPWs were obtained from agricultural soils selected so that they had similar pH but varied primarily in the concentration of DNOM, including fulvic acids and humic acids, although they also differed in major cation and anion concentrations. *Pc*O6 is isolated from field-grown wheat on calcareous soil and promotes plant health under stressed conditions.[7,31] All experiments were conducted with independent triplicate samples in a 2 × 2 × 2 × 4 factorial design with 32 treatments total. The variable factors were the presence/absence of wheat plants, the presence/absence of the probiotic root-colonizing bacterium, *Pc*O6, the presence/absence of CuO NPs, and the type of SPW or the 3.34 mM $Ca(NO_3)_2$. The experimental designs are provided in Tables S1 and S2.† The entire experiment was repeated, to define the variance of test parameters in this biologically active system. Pooling of data within and between studies captures this expected uncertainty in biological systems.

Silica sand supplemented with either a 3.34 M $Ca(NO_3)_2$ solution (termed the "electrolyte" treatment) or one of three SPWs was the growth matrix. The SPWs add complexity to the growth systems[12,13] because they include soluble soil components, although they still lack the complexity of the solid soil matrix. The concentration of 3.34 mM $Ca(NO_3)_2$ was chosen because this ionic strength was within the range of

the SPWs tested. The dose of CuO NPs (100 mg kg^{-1} Cu/sand) was sublethal to wheat while causing substantial root-shortening and increased root exudation.[13] This dose was also sublethal to *Pc*O6 colonized on the wheat root. Controls with Cu^{2+} ions were not tested because the release of Cu^{2+} ions from CuO NPs in the sand matrix varies with time,[12] especially between planted and non-planted sand, and the concentration is difficult to mimic without changing the volume of liquid around the roots.

Preparation of soil pore waters and root-colonizing bacteria

SPWs were generated by saturation paste using deionized water[32] from three soils collected from agricultural sites in Cache County, UT, USA in late summer. The three selected soils are from the same soil series (Millville series, coarse-silty, carbonatic, mesic *Typic Haploxerolls*) and had similar saturated paste pH (7.7 and 7.8), but varied in crop management practices so that DNOM characteristics varied (Table 1). Soil characteristics, the cropping history and cultivation techniques are provided in Table S3.† Characteristics of the SPWs after 0.2 micron filter sterilization are given in Tables 1 and S4.† The SPWs are named after the soil source (OrgM, AgrM, GarM). The GarM SPW was an order of magnitude higher in electrical conductivity than the other SPWs; the top contributors to EC are Ca, K, nitrate, and sulfate. This soil is from a community garden and has been heavily composted for growth of vegetables. Although the higher ionic strength of this SPW may influence NP aggregation and hence dissolution, this soil also contrasts with the other tested SPWs with higher concentration of DNOM, mostly as fulvic acid.

Stock cultures of *Pc*O6 were stored frozen at −80 °C in 15% sterile glycerol until use. Frozen cells were transferred onto a minimal medium agar[33] to grow overnight at 25 °C into the log phase, at which time sterile water was added to the agar surface to create a suspension of cells that was diluted to ~1×10^7 CFU mL^{-1}.

Preparation of wheat seeds, growth boxes and growth conditions

Wheat (*Triticum aestivum* L. v. Deloris, hard red winter wheat) seeds were surface-sterilized in 3% bleach for 10 minutes, then rinsed at least five times in sterile deionized water. The seeds were sown onto Luria–Bertani agar (LB) at approximately 25 seeds per 15 cm–diameter dish. The Petri dishes were sealed with Parafilm® and incubated for four days at 25 °C to permit germination and growth of microbial contaminants (if present).[13]

Growth boxes were prepared as described by McManus *et al.*[13] with some modification. Briefly, silica sand (UMINIC Corp, ID) was washed by hand in deionized water three times, then placed in a 550 °C muffle furnace to oxidize residual organic matter. The sand was washed again with deionized water and dried at 100 °C. One hundred milligrams Cu per kg sand (as CuO NPs) was added to the dry sand and then shaken at high speed on a reciprocal shaker for 30 minutes. The CuO NPs with nominal size of 50 nm (Sigma-Aldrich) have been previously characterized;[6] briefly, the NPs are >99.3% pure, consistent with crystalline tenorite with no surface alterations, the particles have an irregular rounded shapes, an average and median diameter

Table 1 Characteristics of soils and SPWs. Soil samples were collected in 2014 for preliminary experiments in 2015–2016 and tested at the Utah State University Analytical Laboratory under the North American Proficiency Testing Program for Agricultural Labs. Soils were re-collected in 2016 for use in this study. Bolded values are values with an order of magnitude difference from the other SPWs

Soil abbreviation	OrgM	AgrM	GarM
Name origin	Organic farm, Millville	Agricultural field, Millville	Community garden, Millville
Soil series, texture	Millville silt loam	Millville silt loam	Millville silt loam
Particle size distribution (% sand/silt/clay)	19/56/26	22/56/23	13/59/28
Cultivation/crop	Organic certified cover crops and vegetables	Conventional agriculture winter wheat	Unknown amendments, varied crops
Soil pH	7.7	7.8	7.8
Na (mg L^{-1})	11.8	9.4	27.5
Mg (mg L^{-1})	55.7	17.9	146
K (mg L^{-1})	28.7	4.2	**299**
Ca (mg L^{-1})	168	97.4	372
Gluconate (mg L^{-1})	1.9	3.9	**<0.5**
Chloride (mg L^{-1})	50.2	**5.6**	61.6
Nitrite (mg L^{-1} N^{-1})	5.7	11.8	**<0.5**
Nitrate (mg L^{-1} N^{-1})	149	**12.6**	574
Sulfate (mg L^{-1})	36.8	18.4	195
Alkalinity (mg L^{-1} $CaCO_3$)	340	450	490
Electrical conductivity (μS cm^{-1})	735	391	**3380**
Dissolved organic carbon (mg L^{-1} C)	42.7	73.4	**305**
Humic acids (mg L^{-1} C)	<0.8	<0.8	**4.3**
Fulvic acids (mg L^{-1} C)	28.3	38.0	**165**

of 46 and 38 nm, hydrodynamic diameter in deionized water of 318 nm, zeta potential of −9 to −20 mV in 1 mM KCl in the pH range of 6.1–7.5, and zeta potential of −20 mV after incubation in wetted sand with wheat growth. The CuO NPs were kept dry and in dark until use. The sand mixed with the CuO NPs was transferred to acid-rinsed magenta boxes (Sigma-Aldrich, V8505, 10 × 7 × 7 cm) which were closed with lids having the same dimensions as the base, then sterilized at 121 °C for 2 h, then cooled to room temperature. We do not expect the autoclaving process to alter the CuO NPs.[13]

Each box was wetted with either 45 mL of sterile 3.34 mM $Ca(NO_3)_2$ or 0.2 micron-filter sterilized SPWs (OrgM, AgrM, GarM) and mixed with a sterile spatula in a laminar-flow hood. This ratio of water to sand was 1.5 times the sand water content at field capacity.[13] Twenty-five germinated wheat seeds from the agar plates, which showed no signs of microbial infection, were spread evenly into each box and then covered with one-half cm of sterile sand. The lids were secured to the boxes and the seam wrapped in Parafilm® to minimize water losses and air exchange during the growth period. *Pc*O6 was added to specified boxes as 250 μL aliquots of the 1×10^7 CFU mL^{-1} bacterial suspension to each 45 mL aliquot of SPW/electrolyte prior to transfer to the sterile sand in the magenta boxes. Thus, the growth matrix contained about 5.6×10^4 CFU mL^{-1} of *Pc*O6. CuO NPs did not impede *Pc*O6 root colonization as all roots showed colonization by this bacterium irrespective of treatment, agreeing with previous studies.[6,31]

Boxes were set under fluorescent lamps generating a photosynthetic photon flux density of 144 μmol m^{-2} s^{-1} at the box surface for 10 days under a 16 hour light/8 hour dark cycle[12] in a constant temperature room at 25 °C (Fig. S1†). The boxes were randomly rotated daily to minimize light gradient effects.

Harvesting and analytical procedures

Harvesting was performed as described by McManus *et al.*[13] with some modifications. At 14 days after seed germination, the boxes were opened 3–6 h after the beginning light period to ensure maximum root exudation.[34] Forty-five mL of sterile deionized water was added to each box and allowed to equilibrate for 15 minutes, bringing the total volume of added liquid to 90 mL. Wheat seedlings were gently removed from the sand and one root for each treatment was placed on a sterile LB agar to test for culturable microbes. The sand was mixed and placed in a sterile, acid-rinsed glass funnel to extract the rhizosphere solution (RS) by vacuum. The RS is defined as the SPW or 3.34 mM $Ca(NO_3)_2$ modified by plant growth and *Pc*O6. Fifteen μL of RS was immediately placed on an LB agar to test for bacterial contamination and/or verify the presence of *Pc*O6. The *Pc*O6 appeared in all *Pc*O6 treatments and did not appear in sterile treatments. The roots of the plants were rinsed with deionized water to remove attached sand, the mean root length was measured, and then the shoots and roots were sectioned. The coleoptile portion of the shoot (about 3 cm of shoot above the seed) was discarded due to potential NP contamination.[31]

The pH and electrical conductivity of the RSs were measured by standard methods.[35] Centrifugation at 20 800 × *g* for 15 minutes was used to remove NPs >30 nm in diameter (Eppendorf rotor F45-30-11, Eppendorf centrifuge 8504).[13] Low molecular weight organic acids (LMWOAs) and inorganic anions in the RSs were measured by ion chromatography (Dionex method 123, Dionex ICS-3000). Cations, including Cu, were measured by ICP-MS (Agilent 7700x) with USEPA method 6020 and dissolved organic carbon was assessed using a combustion carbon analyzer (Teledyne Tekmar Apollo 9000) by standard methods.[35] The phytosiderophore, DMA, in the RS was measured using liquid chromatography triple quadrupole mass spectrometry (Agilent 1290 ultra HPLC, Agilent 6490),[13] and the pyoverdine-like siderophore from *Pc*O6 was measured by fluorescence (BioTek, Inc. Synergy4 Hybrid Multi-Mode Micro plate reader).[30] Free (non-complexed) Cu^{2+} was measured with a Cu ion-selective electrode (Orion 96-29 Ionplus).[36] Metal contents in the roots, shoots, and sand were measured by ICP-MS after hot nitric acid digestion.[37,38]

To back-calculate the concentrations of constituents in the RSs, the boxes were weighed at the beginning and end of the growth period (10 days). Boxes typically lost between 0–2 g water. Roots and shoots were weighed before and after drying to calculate the water content of the plant. The following formula was applied to calculate a dilution factor:

$$\text{Dilution factor}_{\text{box\#}n} = \frac{(90\ \text{mL} - (\text{water loss from box/plant}))}{(45\ \text{mL} - (\text{water loss from box/plant}))} \quad (1)$$

Net root elongation assays

The bioavailability of differing Cu-ligand complexes to wheat roots was reported through a net root elongation assay using 2 day wheat seedlings with an average beginning root length of 7.5 mm. The method was based on previous root elongation assays with some modification.[20,21,25,26] Six wheat seedlings were floated on plastic mesh rafts on 1 L sterile solutions in triplicate sterile beakers with Cu-ligand complexes buffered at pH 6.75 by 1 mM MOPS (adjusted with small amounts of trace metal grade nitric acid or sodium hydroxide). Solutions were maintained for 2 additional days, replacing the solution with fresh solution after 1 day to reduce wheat- or microbial-induced changes in the solutions. The pH was chosen to be as close to the RSs as possible without precipitating the dissolved Cu. The Cu, added as $Cu(NO_3)_2$, in all solutions was maintained at 1.6 μM, and the ligand concentration was varied to complex as close to 100% of the Cu as possible based on geochemical modeling while remaining near the previously observed ligand concentration ranges measured in the RSs. Control treatments, with the ligand but without Cu, were also included. The pH and concentrations of Cu and ligand were checked every 24 h. At 2 d of growth in the solutions, the root length was measured

and the net root elongation for each Cu-ligand treatment was calculated using eqn (2). Due to limited availability (prohibitive cost) of the ligand DMA, the test volume was reduced from 1 L to 50 mL for DMA. The no-ligand, citrate, and malate treatments were also repeated in 50 mL test volumes to ensure consistency. There was no difference in net root elongation results for the no-ligand, citrate, and malate tests in 50 or 1000 mL.

$$\text{Net root elongation} = \frac{\left(\text{Root length}_{\text{Cu-Ligand,48h}} - 0.75\ \text{cm}\right)}{\left(\text{Root length}_{\text{Ligand,48h}} - 0.75\ \text{cm}\right)} \quad (2)$$

Statistical analysis

JMP 8 (SAS Institute) was used as the statistical package software in all analyses. Plant responses were first analyzed by Student's *t*-test between pooled samples with and without CuO NPs to determine if the NPs caused a toxic response (root length, shoot length, uptake of Cu and other metals into shoots and root, root exudate production). If a toxic response was found, then two-way ANOVA with presence/absence of the factors *Pc*O6 and RS type was conducted only on samples with CuO NPs (8 of the 32 treatments) to determine how the SPWs and bacteria affected the plant response to the CuO. *Post hoc* Tukey honestly significant difference test or Student's *t*-test were used to determine significant differences among the two factors *Pc*O6 and RS type, and their interaction, in the samples ($\alpha = 0.05$). Square root and logarithmic transformations were necessary in some variables to maintain normal distribution of the residuals. Measurements shown in graphs and provided in the text are the average of pooled replicates from the two independent studies ($n = 6$) with significant differences shown and 95% confidence intervals to display the distribution of the data unless noted. Agreement between the two independent studies was evaluated by the 16 variables shown in figures throughout the text using *F*-tests. Four of the 16 variables were different between the studies: DMA ($F = 0.0014$), root Cu ($F = 0.0402$), shoot length ($F = 0.0073$), and root length ($F = 0.0474$); these factors are expected to reflect normal biological variability. Root Cu is also a particularly difficult variable to measure due to adsorption of sand grains and CuO NPs to the root. Despite the differences between trials for these parameters, the response trends within both trials remained the same and included the error necessary to define the variance of this biologically active system.

A chi-squared test ($\alpha = 0.05$) was used to determine if microbial contamination was evenly distributed across all treatments.

Principal components analysis was used to determine relationships among variables in wheat and in RSs. Variables included in the principal components analysis were only included if <50% of the measurements were censored (*i.e.*, the censored data could be imputed). Censored data were imputed from a normal distribution of the non-censored data. To confirm relationships among variables, the significance and magnitude of the pairwise Pearson's correlations was considered.

Data from the root elongation assays were analyzed by comparing the net root elongation of each Cu-ligand treatment to the net root elongation of the free Cu treatment by using Dunnett's test ($p < 0.05$).

Geochemical modeling of the solution phase in the rhizosphere was performed with Visual MINTEQ v3.1 using inputs detected at harvest, an imposed crystalline tenorite (CuO) solid phase, a closed atmosphere, and precipitation disallowed. Data for metal–DMA and metal–gluconate complexes were added to the database as previously described.[13]

Results and discussion

Wheat growth responses to CuO NPs

First the data were compared to determine if the CuO NPs, regardless of treatment, influenced wheat biological parameters. Root length was significantly shortened by growth with CuO NPs (100 mg kg^{-1} Cu/sand) by an average of 37.2% compared to the non-CuO NP control (Fig. 1). Root shortening in wheat has been observed previously upon growth with both CuO NPs[12,13] and Cu ions for winter wheat[12] and durum wheat.[39] Shoot length was significantly decreased by an average of 3.4% overall (0.6 cm) by CuO NPs ($p = 0.011$, Fig. 1). The finding that shoot length was minimally affected by CuO NPs concurred with previous research showing CuO NPs decreased wheat shoot length by 13% at a dose of 500 mg kg^{-1} Cu[12] but not at lower doses (0–300 mg kg^{-1} Cu).[13]

Shoot and root Cu levels increased 2.0-fold and 177-fold, respectively, in wheat exposed to the CuO NPs (Fig. 1). Shoot length and Cu concentration were less sensitive to CuO NPs than root length and Cu concentration, and may relate to sequestration of Cu in the root cell wall as discussed by Peng *et al.*[40] for *Elsholtzia splendens* (mint family), Kopittke *et al.*[41] for cowpea, and Meychik *et al.*[42] for wheat. Sequestration of Cu from CuO NPs in wheat roots resulting in little change to shoot Cu and length across a range of CuO NP dosing was seen in previous studies.[13,14] We also observed with sand-grown wheat that some of the Cu associated with the roots was due to surface depositions of NP aggregates and sand.[6,27,43] CuO NPs proliferate root hairs which accounts for these depositions.[27] Water rinsing did not remove all the sorbed, agglomerated particles. The contents observed in these sand-grown roots were similar to those reported from doses of 50 and 100 mg L^{-1} CuO NPs respectively obtained from hydroponic studies with wheat.[43] Zhou *et al.*[43] estimated 29–34% of the root Cu was surface-adsorbed NPs rather than being internalized metal under their conditions and we assume this estimate is applicable here, meaning that root Cu without adsorbed NPs was closer to ~183 mg kg^{-1}.

Shoot K, shoot Fe, and root Mn were decreased 21%, 8.7%, and 34% respectively by the presence of CuO NPs

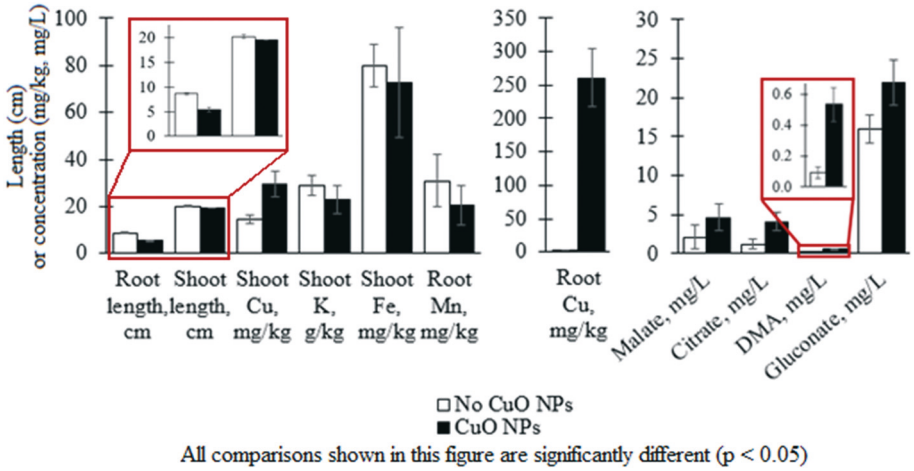

Fig. 1 Root and shoot length and tissue accumulation of Cu and other metals and metabolites in the RS in the no CuO NPs (solid white) and CuO NPs (solid black) treatments. All comparisons shown between no CuO NPs and CuO NPs are statistically different ($\alpha < 0.05$) by Student's *t*-test. Non-significant comparison are not shown. The error bars show 95% confidence intervals (n = 48 per bar) to illustrate the spread of data, but do not determine significant differences. Root and shoot metals required a logarithmic transformation to maintain normal distributions of residuals.

compared to non-CuO NP exposed plants (Fig. 1). Depression of shoot K, shoot Fe, and root Mn by CuO NPs has been previously observed[13] and decreased Fe was found for durum wheat grown hydroponically exposed to Cu^{2+}.[39] The decrease in shoot K could be due to greater root K export as a stress response, while lower shoot Fe is thought to be due to competition of dissolved Cu for the Fe phytosiderophore DMA.[13] Changes in accumulation of shoot Ca/Mg/Mn and root Ca/Mg/K is reported for wheat growth with CuO NPs[13] but were not observed here. Ca, Mg, K, and Mn were supplied by the SPWs (Tables 1 and S4†) in greater levels than for previous growth studies utilizing only 0.7 mM $Ca(NO_3)_2$ or DDW, minimizing the detrimental effect of CuO NPs on wheat roots. This improved supply of essential metals could have reduced the extent of inhibition of root elongation. These results imply that plant nutrition will be less affected by CuO NPs when the plants are grown in soil rather than the sand or hydroponic matrix of laboratory studies.

RS dissolved organic carbon was not altered by the presence of CuO NPs in this study (data not shown) although there were changes in the composition of the exudates. Malate, citrate, and DMA in the RSs increased when CuO NPs were present (Fig. 1). These increases were consistent with previous reports for plants grown in sand and water matrices or hydroponic systems in response to CuO NPs[13] and Cu ions.[44] Increased release of malate and citrate, a documented response to Cu toxicity,[44] is hypothesized to be protective[13] as complexation of Cu ions with malate and citrate partially or fully relieves Cu toxicity to wheat roots.[20] The reason for an increase in DMA is unclear, although DMA is a strong Cu chelator. Another potential complexing agent, gluconate, also increased in response to growth with CuO NPs. The origin of gluconate in this system is unknown but could be produced by bacterial endophytes not eliminated by surface sterilization.[13]

These results confirmed that CuO NPs at the 100 mg kg^{-1} Cu level had an impact on root length, modified the metal contents of shoot and root tissues and changed the composition of the root exudate. Soil compounds in the RSs did not fully eliminate the toxic response.

SPWs and *Pc*O6 changed root length in CuO NP exposed wheat

Having established a general toxic response of CuO NPs, we next examine the effects of the individual SPWs and root-colonizing bacteria on the wheat responses to CuO NPs. CuO NPs reduced root length under all RSs compared to plants grown without NPs (Fig. 2A and S2†), but roots were less stunted in the presence of SPWs (averaging 26–35% shorter) than the electrolyte control (59% shorter). Inhibition of root elongation by CuO NPs was about two-fold greater than the 33% decrease observed by McManus *et al.*[13] when 0.7 mM $Ca(NO_3)_2$ was used at the same NP dose in sand. For growth under the same conditions, but without electrolyte, there was about a 65% decrease in length.[31] Shoot length was enhanced with the GarM RS over the other treatments (Fig. 2B).

Although DNOM is proposed to limit toxicity of CuO NPs through binding free Cu^{2+} ions, root elongation was highest with the AgrM RS rather than the GarM RS with the highest DNOM (Tables 1 and S4†). Likewise, higher electrical conductivity may increase competition between Cu^{2+} and other cations for root binding sites, and/or induce NP aggregation (not measured), reducing NP solubility, thus reducing toxicity in the GarM RS; but this was not observed. Therefore, other SPW factors were active. As roots grown in GarM and OrgM RSs without CuO NPs were shorter than AgrM and electrolyte RSs without CuO NPs (Fig. 2A, horizontal bars), we propose that greater available nutrients in OrgM and GarM (Table 1) were responsible. The higher electrical conductivity of the GarM SPW, in contrast,

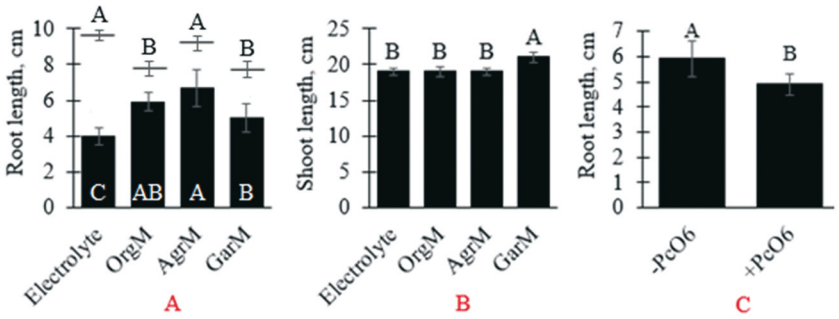

Fig. 2 Root length (A) and shoot length (B) as affected by RS, and root length as affected by *Pseudomonas chlororaphis* O6 (*Pc*O6) (C) in the presence of CuO NPs (100 mg Cu per kg). Shoot length was not affected by *Pc*O6 and is not shown. The error bars show 95% confidence intervals (n = 12 per bar in A and B and 24 per bar in C) to illustrate the spread of data, but do not determine significant differences. Bars with differing letters (A and B, *etc.*) are statistically different ($p < 0.05$) by Tukey's honestly significant difference or Student's t-test after two-way ANOVA. The horizontal bars in graph A show the root length of the non-CuO treated wheat. In graph A, the black letters only apply to horizontal bars, and the white letters only apply to vertical bars; there is no equivalency between the black and white letters.

did not seem to inhibit plant health or response to NPs. Increased electrical conductivity alone did not decrease the GarM SPW root length more than the OrgM SPW, nor did it decrease shoot length relative to the other SPWs (Fig. 2).

*Pc*O6 reduced root elongation in the presence of CuO NPs (Fig. 2C). While *Pc*O6 colonization changes root morphology to be more robust,[31] it is possible that the production of indole acetic acid (IAA) by *Pc*O6 contributed to the root shortening.[27] Production of IAA by *Pc*O6 is increased by CuO NPs in liquid cultures.[45] This process may compound the effects of CuO NPs on IAA function at the root tip, where reduction in elongation but proliferation of elongated root hairs due to Cu-responses has been demonstrated.[27] Furthermore, it is reported that Mn oxidation occurs with *Pseudomonas putida* isolates and other *Pseudomonas* isolates,[46] but the metabolic responses of *Pc*O6 to oxidation/reduction of metals ions such as Cu and Mn are not known but could alter the bioavailability of Cu to wheat. There was no interactive effect of RS and *Pc*O6 on root length.

Essential metals

Overall, metals in the wheat tissue, including Cu, did not vary greatly in response to wheat seedling growth with the SPWs or bacterial colonization; the exceptions are discussed here. Root Cu content did not vary with RS, but was decreased by *Pc*O6 colonization (Fig. S3†), dropping from an average of 325 mg kg^{-1} dry weight for the plants exposed to CuO NPs without *Pc*O6 to 197 mg kg^{-1} dry weight for plants grown with both CuO NPs and *Pc*O6. It is possible that the formation of the biofilms overlying the root surface and metabolites secreted by *Pc*O6 affected Cu root uptake.[6] Root Mn and shoot Fe were neither significantly affected by RS or *Pc*O6 (data not shown), despite the overall tissue concentration changes caused by addition of CuO NPs (Fig. 1).

Shoot Cu did not vary greatly in this study (Fig. S3†), nor in soil-grown wheat when exposed to different labile Cu concentrations from CuO NPs.[14] A general toxicity threshold for all plants for shoot Cu is around 30 mg kg^{-1} Cu/dry shoot;[47] whereas, a lower threshold value, 17 mg kg^{-1} Cu/dry shoot, is reported for wheat.[48] Although the Cu shoot levels measured in these studies were all above this 17 mg kg^{-1} Cu/dry shoot threshold value (Fig. S3†), there was limited impact on shoot elongation under these growth conditions (Fig. 1), perhaps due to cultivar and age differences. Thus, the protection against root shortening seen in the interactions with three RSs from SPWs and CuO NPs could not be explained by specific SPW influences on root or shoot Cu content.

Shoot K was affected by RS but not *Pc*O6 (Fig. S3†); shoot K was highest in the GarM RS relative to the other three RSs, reflecting the 10-fold higher K concentration in GarM (Table 1). A similar pattern to shoot K was seen for shoot Mg and Ca, although these secondary macronutrients were not impacted by CuO NPs, again demonstrating that elevated cation primary and secondary macronutrient availability in the GarM RS played an important role in plant health during CuO NP challenges.

Metabolites in the rhizosphere solutions

A two-way ANOVA showed that citrate and malate levels were dependent on the interaction of *Pc*O6 and the RS (Fig. 3A and B). Citrate and malate were reduced in the RSs from all *Pc*O6-inoculated growth boxes to below the detection limit (0.5 mg L^{-1}) and agreed with findings for wheat growing in a sand and water system.[31] These organic acids are preferred carbon sources for pseudomonads. Gluconate was reduced but not eliminated by *Pc*O6 (Fig. 3E) and pathways for both anabolism and catabolism are expected from the genome of this microbe. Malate was elevated in the AgrM RS compared to the electrolyte and other RSs, while citrate and gluconate was elevated in the OrgM and GarM RSs compared to the electrolyte. These differing responses may be partially explained by the chloride content of the SPWs. Chloride is an osmoticum that may replace malate[49] and is shown to reduce malate formation in the epidermis of *Vicia faba*.[50] Thus chloride in the OrgM/GarM SPWs (Table 1) could have

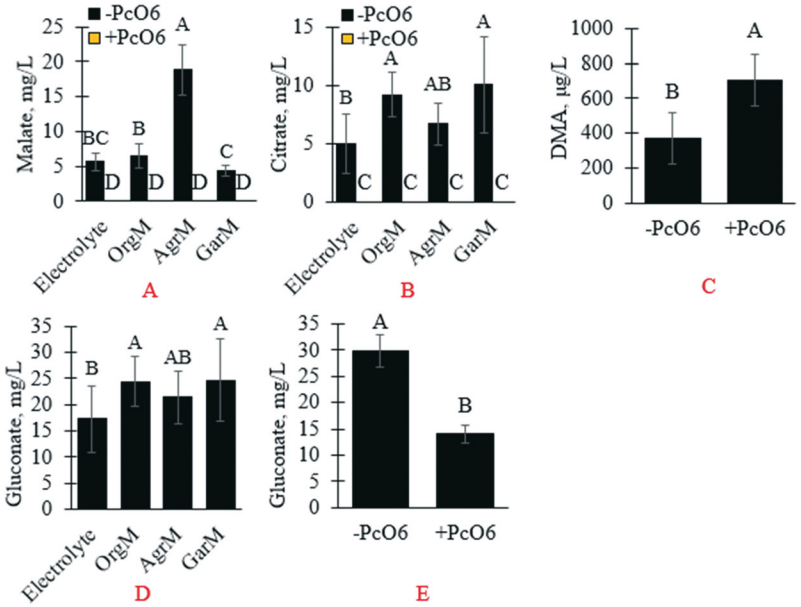

Fig. 3 Malate (A), citrate (B), DMA (C), and gluconate (D and E) in RSs from planted samples with CuO NPs. Graphs A and B show the interaction of RS and *Pseudomonas chlororaphis* O6 (*Pc*O6), C and D show the effect of RS, and E shows the effect of *Pc*O6. When *Pc*O6 was present, detectable citrate/malate levels fell below detection. The error bars show 95% confidence intervals (n = 24 (C and E), 12 (D), or 6 (A and B) per bar) to illustrate the spread of data, but do not determine significant differences. Bars with differing letters (A and B, *etc.*) are statistically different ($p < 0.05$) by Tukey's honestly significant difference (A, B and D) or Student's t-test (C and E) after two-way ANOVA.

reduced malate in the root and the exudates. Altered malate exudation in wheat is also caused by Al[44] or Cu toxicity.[13,44] Decrease in malate and other root exudates alters the extent of Cu complexation and thus impacts root stunting. However, currently the effects of low chloride concentration on wheat root exudation is not studied. Regardless, the differing root and bacterial exudate profiles will have implications for the transformation and dissolution of CuO NPs.

DMA was not affected by differing RSs, but was increased by colonization of *Pc*O6 (Fig. 3C). DMA is reported to be degraded in bulk soil environments, primarily by Gram-negative bacteria, such as pseudomonads, with a half-life of 3–8 h similar to other LMWOAs.[51] Thus, the persistence of DMA in our system at increased levels with *Pc*O6 presence is unanticipated but could be explained by the time of harvest and the competition for Fe between bacteria and plants. The plants were harvested three hours after photosynthesis began, the peak of DMA production.[34] Also, a Student's t-test between the *Pc*O6 and non-*Pc*O6 samples showed that *Pc*O6 reduced soluble Fe in the RSs from 188 μg L^{-1} to 138 μg L^{-1} on average. Examination of the RSs for fluorescence typical of the unloaded pyoverdine-like siderophore produced by *Pc*O6 under Fe limitations did not reveal its presence (data not shown). The lack of pyoverdines may be explained by the observation that Fe measured at the end of the study (163 μg L^{-1} on average) was not below the level generally required to stimulate pyoverdine production (~56–100 μg L^{-1}).[52] Pyoverdine production may also have been suppressed by the presence of CuO NPs as was observed for planktonic *Pc*O6 cultures.[30,52] Thus the Fe level was sufficient for *Pc*O6, not requiring production of pyoverdines, but insufficient for wheat, which may have produced more DMA to scavenge additional Fe.

Cu solubilization from CuO NPs

While the transformations of CuO NPs in the rhizosphere was not the focus of this study, it is important to consider the contribution of dissolved metals in plant toxicity. Dissolved Cu detected in the RSs after plant harvest with CuO NPs without *Pc*O6 was between 4000 and 6000 μg L^{-1} with no significant differences between growth with electrolyte and the different SPWs (Fig. 4A). The SPWs contributed less than 50 μg L^{-1} Cu to the RSs (Table S4†). The dissolved Cu was lowest in the OrgM RS, which had the lowest humic and fulvic acid content, both of which will complex soluble Cu (Table S4†). However, the difference in soluble Cu did not influence Cu root content (Fig. S3†). These solubilities are generally higher than what is reported in whole soils.[17] Soils introduce large surface areas of minerals and solid organic matter which will sorb Cu^{2+} ions and NPs, removing them from solution.

While the concentrations of dissolved Cu were mostly equivalent in the RSs (Fig. 4A), uncomplexed Cu^{2+} ions differed. Free Cu^{2+} ion concentration was reduced in the RSs by the increasing DNOM content compared with the levels in the electrolyte RS (Fig. 4B). Based on the differences seen in free Cu^{2+} between the electrolyte and SPWs, we propose that the metabolites in the root exudates did not complex Cu as effectively as the components of DNOM from SPWs, such as the humic and fulvic acids. Inorganic anions varied greatly

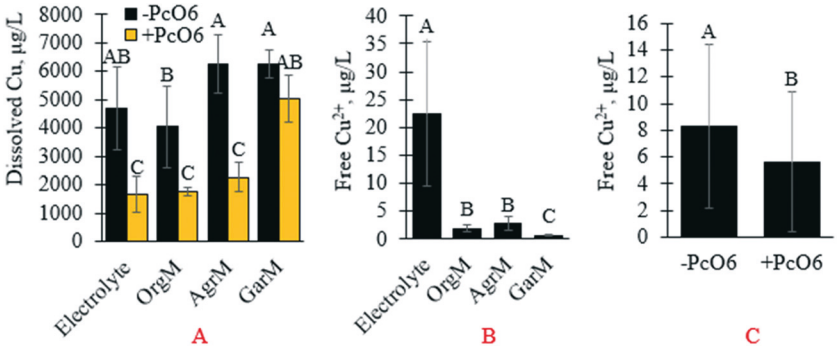

Fig. 4 Dissolved Cu (A) as affected by the interaction of RS and *Pc*O6 and free Cu^{2+} as affected by RS (B) and *Pseudomonas chlororaphis* O6 (*Pc*O6) (C) in planted samples with CuO NPs. The error bars show 95% confidence intervals (n = 6 (A), 12 (B), or 24 (C) per bar) to illustrate the spread of data, but do not determine significant differences. Bars with differing letters (A, B, *etc.*) are statistically different ($p < 0.05$) by Tukey's honestly significant difference (A and B) or Student's t-test (C) after two-way ANOVA. Free Cu^{2+} required a logarithmic transformation to maintain normal distributions of the residuals. When CuO NPs were not included, the RSs had an average of 50.6 ± 11.4 µg L^{-1} dissolved Cu and 0.30 ± 0.09 µg L^{-1} free Cu^{2+} (95% confidence intervals).

among the SPWs (Table 1) and did not significantly complex Cu by MINTEQ analysis.

*Pc*O6 colonization lowered both dissolved Cu and the free Cu^{2+} (Fig. 4A and C). With *Pc*O6, the dissolved Cu was reduced to less than 2.3 mg L^{-1} when the plants were grown with the electrolyte, OrgM, and AgrM RSs, a finding that could explain the reduction in root Cu with *Pc*O6 (except for the GarM RS). The GarM SPW had the highest humic and fulvic acid values (Table 1) which could explain the lack of effect of *Pc*O6 on dissolved Cu.

Contaminating bacteria in the rooting zone

Bacterial contamination in sterile planted systems is not commonly discussed in the literature yet their metabolism could have important consequences in the fate of the CuO NPs provided to the growing root. In this study, we found 60 out of 192 samples from the RSs (31%) tested positive for culturable bacteria other than *Pc*O6 (Table S5† and additional discussion). Only one growth box with *Pc*O6 was contaminated. Although *Pc*O6 eliminated citrate and malate in the RSs of planted systems, the contaminating bacteria apparently did not deplete these metabolites. The contaminating bacteria also did not significantly metabolize or produce dissolved organic carbon or alter pH (Fig. S4†) in the RSs. *Pc*O6 root colonization decreased dissolved organic carbon (181 mg L^{-1} C to 135 mg L^{-1} C) and increased pH (7.54 to 7.78) as shown by Student's t-test. These lines of evidence suggested the influence of the contaminating bacteria was minimal for the parameters studied for this work.

Mechanism of SPW protection against NPs: the biotic ligand model does not explain observed root toxicity

All RSs from SPWs remediated the inhibition of root elongation by CuO NPs compared to growth with the electrolyte RS (Fig. 2A). In order to determine whether the hypotheses of the biotic ligand model were upheld (roots should be longest at high pH/DNOM/high Ca^{2+}, Mg^{2+} for equal concentrations of dissolved Cu),[21,25,26] a principal components analysis was performed with the data obtained from growth with and without CuO NPs.

Data from the RSs with CuO NPs separated from the data without CuO NPs mainly along the first principal component axis (Fig. 5A). The GarM RS samples also grouped away from the data of other RSs (electrolyte, ArgM, OrgM) along the second principal component axis. These findings suggest that the GarM RS has substantially different ionic strength, pH, and DNOM properties (Fig. 5B) from the other RSs, factors consistent with the chemical analysis of the SPWs (Tables 1 and S4†). Although the higher Ca, K, sulfate, and nitrate content of the GarM RS, contributing to the EC, may alter the aggregation of the NPs (which was not investigated in this study), the solubility of the NPs in this RS was not different from the AgrM or electrolyte RSs (Fig. 4A). The free Cu^{2+} measured in the GarM RS follows the increasing DNOM pattern (Fig. 4B). Dissolved Cu correlated with DNOM (as measured by dissolved organic carbon) but not electrical conductivity (Table S6†). Thus, DNOM, but not electrical conductivity, was a differentiating factor in CuO NP solubility.

Two main groupings of variables were observed. Shoot length was grouped with pH, electrical conductivity, and dissolved organic carbon (inversely with malate and free Cu^{2+}). Both root and shoot Cu were grouped with dissolved Cu, citrate, and gluconate (Fig. 5B). This latter group was inversely associated with root length. In other words, shorter root length was associated with increasing concentrations of root Cu, dissolved Cu, gluconate, and citrate.

Pearson's correlations (Table S6†) confirmed these associations. The strongest significant correlations for root length were negative association with free Cu^{2+} (R = −0.549) and dissolved Cu (R = −0.571), suggesting that Cu complexes as well as free Cu^{2+} played a role in wheat response. Root Cu was positively associated with the wheat and *Pc*O6 metabolites, gluconate (R = +0.601), citrate (R = +0.522), and

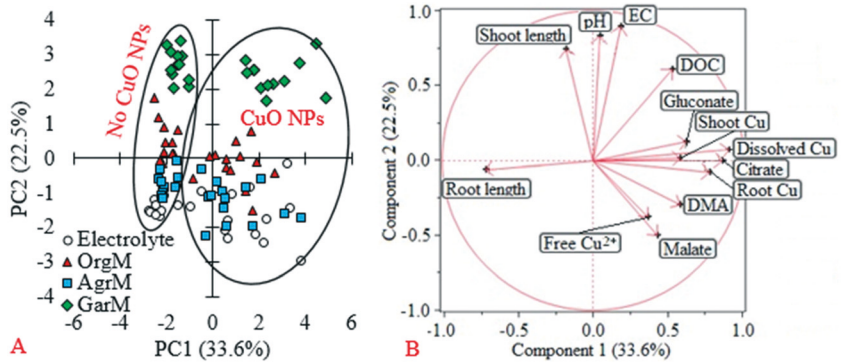

Fig. 5 Principal components analysis score plot (A) and loading plot (B) of all planted samples in study with and without *Pseudomonas chlororaphis* O6 (*Pc*O6). Score plot values for *Pc*O6 samples were imputed by JMP. Variables included (13) were gluconate, malate, citrate, DMA, dissolved organic carbon (DOC), pH, electrical conductivity (EC), dissolved Cu after centrifugation, free Cu^{2+}, root Cu, shoot Cu, root length, and shoot length. Fulvic and humic acids were not included in the principal components analysis because they were only measured in the SPWs and not the RSs. PC = principal component.

DMA (R = +0.559), and, consequently, the dissolved Cu (R = +0.791) further suggesting bioavailable Cu complexes were present. Dissolved Cu correlated positively with gluconate (R = +0.656), citrate (R = +0.720) and DMA (R = +0.508) showing that CuO NP solubility was enhanced by root and *Pc*O6 exudates. Shoot Cu was negatively associated with root length (R = −0.598) indicating the strong influence of root functions with the shoot. As roots showed increased Cu content, the shoot Cu concentrations also increased, though tightly regulated by retention in the rooting zone.[13]

The correlations revealed by the principal components analysis (Fig. 5A and B) and Pearson's correlations (Table S6†) did not fully support the hypotheses of the biotic ligand model. Competing cations (shown as electrical conductivity/pH) and dissolved organic carbon were associated with increasing shoot length but not root length, which could indicate some protection being derived from ligands and competing cations. While free Cu^{2+} was significantly associated with shortened roots, dissolved Cu was also an effector. Furthermore, the wheat responses to Cu in these growth conditions were more intense than other studies using free Cu^{2+}. In hydroponic conditions, Michaud *et al.*[39] observed a 50% wheat root length reduction after 8 d at 38 μg L^{-1} free Cu^{2+} and a similar root length reduction of 59% was seen in the electrolyte RS with a free Cu concentration of 22.5 μg L^{-1} after 14 d. However, Michaud *et al.*[39] observed a 25% reduction of wheat root length at 12.7 μg L^{-1} free Cu^{2+}, and here, a wheat root length reduction of 26–35% was seen at just 0.64–2.83 μg L^{-1} free Cu^{2+}. This observation suggests that some Cu complexes were also bioavailable.

Bioavailable Cu complexes played a role in inhibition of root elongation

The findings of the positive association of root Cu with gluconate, citrate and DMA, and a negative association of root length with these same factors, suggested that some Cu-complexes in the RSs were bioavailable to the wheat. Some bioavailable Cu complexes are known: Cu–malate was previously found to be bioavailable to wheat,[20] as well as some inorganic Cu complexes.[21] Organic complexes of Cu may also be involved in root to shoot transport of Cu. Complexes of Cu with citrate as well as asparagine and histidine were primary forms for xylem transport of Cu and Zn in soybean and tomato.[53] The Cu–DMA complex has been detected in rice xylem.[54] While we did not determine the amino acid composition of the RSs for this paper, several amino acids are reported to be released in wheat root exudates.[13] The bioavailability of Cu–humic acid or fulvic acid complexes seems little-researched, but one study found that humic acid lengthened wheat roots under Cu stress.[55]

To investigate the bioavailability of differing Cu-ligand complexes to wheat, wheat root elongation assays were conducted with 1.6 μM Cu^{2+} and various metabolites as ligands. The study used ligands at a concentration that was both similar to levels previously measured in the rhizospheres, and also sufficient to complex as close to 100% of the free Cu^{2+} as possible (Table 1). The ligands were chosen based on the significant components seen in this study as well as amino acids which were not measured in this study, but which are strong Cu ligands and were previously observed in root exudates.[13] The pH was set to 6.75 as pH > 6.75 would theoretically precipitate the free Cu^{2+} in the no-ligand assay. The speciation of the test solutions was calculated using MINTEQ.

We found that certain Cu-complexes inhibited root elongation, as was observed with free Cu, whereas others did not. Complexation of Cu with citrate and fulvic acid partially mitigated the inhibition of root elongation of the wheat seedlings when compared with the response to 1.6 μM free Cu^{2+} toxicity (Table 2). However inhibition of root elongation was observed with the Cu-ligands of malate, DMA, arginine, valine, and glycine. Our findings of Cu–DMA bioavailability in particular are supported by the observation that DMA increased Cu uptake into wheat in some Cu-contaminated soils.[56]

Table 2 Net root elongation of 4 day-old wheat seedlings after 48 h exposure to mixtures of 1.6 uM Cu^{2+} and 2–200 μM ligands. Net root elongation values are given as the average plus or minus the standard error of the mean (SEM). Values with an * are significantly different from the no-ligand control by Dunnett's test ($p < 0.05$). Values with a † are those measured in this study. Raw data is shown in Fig. S5

Ligand, μM (mg L^{-1})	Free Cu^{2+} (%)	Cu-Ligand (%)	Net root elongation (% ± SEM)	Previous rhizosphere ligand concentrations
No ligand	85.0	15.0	21.2 ± 6.1	Not applicable
Citrate, 50 μM (9.5 mg L^{-1})	0.10	99.9	69.5 ± 10.0*	7.3–48.1 mg L^{-1} (ref. 13) 1.4–15.8 mg L^{-1}†
Malate, 200 μM (26.4 mg L^{-1})	14.9	81.8	19.6 ± 8.3	22.1–71.1 mg L^{-1} (ref. 13) 3.5–25.4 mg L^{-1}†
Gluconate, 200 μM (39.0 mg L^{-1})	20.5	74.9	29.6 ± 2.3	16.7–114 mg L^{-1} (ref. 13) 4.0–34.8 mg L^{-1}†
DMA, 2 μM (0.6 mg L^{-1})	0.0	100	2.9 ± 4.2	0.44–3.94 mg L^{-1} (ref. 13) 0–1.36 mg L^{-1}†
Arginine, 50 μM (8.7 mg L^{-1})	0.59	99.3	10.1 ± 5.6	0.38–1.56 mg L^{-1} (ref. 13)
Valine, 50 μM (5.8 mg L^{-1})	3.5	95.7	17.7 ± 7.2	0.24–1.6 mg L^{-1} (ref. 13)
Glycine, 50 μM (3.7 mg L^{-1})	3.8	95.4	16.9 ± 5.4	0.07–1.5 mg L^{-1} (ref. 13)
Fulvic acid, 50 μM C (0.6 mg L^{-1})	0.0	100	62.1 ± 8.0*	42.7–165 mg L^{-1}†
Humic acid, 50 μM C (0.6 mg L^{-1})	0.5	99.4	15.1 ± 2.9	0–4.3 mg L^{-1}†

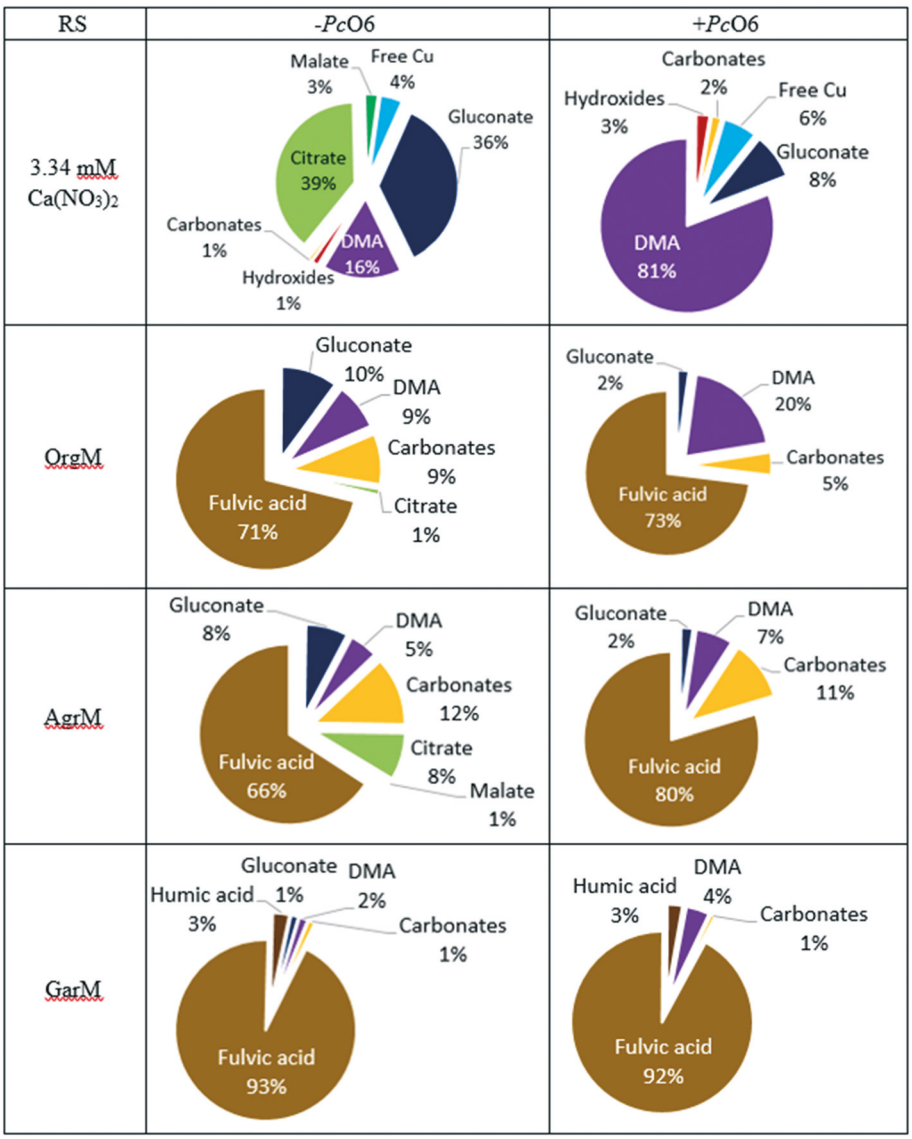

Fig. 6 Geochemical model predictions of distribution of dissolved Cu complexes in planted samples with CuO NPs across all RSs and *Pseudomonas chlororaphis* O6 (*Pc*O6) conditions.

There was 2.5–3 times more growth in the humic acid and gluconate roots over the control (without Cu), indicating a stimulating effect of these compounds or from the Ca associated with the gluconate (Fig. S5†). Cu–humic acid and Cu–gluconate resulted in roots that had a greater root length than the free Cu treatment (Fig. S5†), but similar net root elongations compared to the Cu-complexes with malate, DMA, arginine, valine, and glycine.

Because dissolved Cu-complexes, and not just free Cu ions, were associated with toxic wheat responses to CuO NPs, the complexation of Cu with different ligands measured in the RSs was investigated using geochemical modeling. The fulvic and humic acid values were entered into the geochemical model based on the SPW measurements before wheat growth. It is possible but unlikely that their functional levels in the RSs differed due to degradation or sorption to the growth matrix sand particles during the course of plant growth. The root exudates were the only organic species to form Cu complexation in the electrolyte RS; thus, Cu is predicted to be complexed by citrate, gluconate, and DMA (Fig. 6) which have large differences in the root elongation assay. The electrolyte RS also had the highest free Cu^{2+}, Cu–malate, and Cu-hydroxides, all of which appear to be bioavailable in the root bioassay and other sources.[20,21] Root colonization by *Pc*O6 (Fig. 6) changed the complexation pattern with Cu–DMA becoming dominant. The bioavailability of this complex provides another potential explanation for the enhanced inhibition of root elongation in the presence of *Pc*O6, in addition to the IAA hypothesis discussed earlier.

In the presence of SPW, Cu–fulvic acid complexes dominate accounting for over 66% of the complexed Cu with *Pc*O6 having limited influence on the distribution. The GarM RS was the only sample with any Cu–humic acid complexes. These findings are interesting based on the observation that Cu complexed with fulvic acid had limited bioavailability in the root elongation assay (Table 2). Thus, we propose that the extensive complexation of Cu as dissolved fulvic acid complexes in the RSs from SPWs allowed greater root elongation in the presence of the CuO NPs compared to the electrolyte because of lesser Cu bioavailability. Overall, Fig. 6 clearly shows that the order of Cu complexing efficiency in the rhizosphere is inorganic anions < citrate/malate/gluconate < fulvic/humic acids < DMA. This finding is supported by the same general order of increasing magnitude of their stability constants with Cu.

In addition to binding free Cu ions,[22] the humic and fulvic acids may coat NPs. Coating with these acids and other organic matter present in soils and natural waters is documented.[23,24,57] In previous studies, coatings lowered CuO NP toxicity through steric repulsion between NPs and rice roots[58] or the cells of the bacterium, *Escherichia coli*.[24] The coating aspect of NP transformation was not investigated in this study.

In the principal components analysis (Fig. 5), the measured concentrations of gluconate, citrate, and DMA were most strongly associated with both dissolved Cu and root Cu, and inversely associated with root length. The principal components analysis showed malate was not a factor associated with root length despite previous evidence showing bioavailability of Cu–malate to wheat roots (Table 2);[20] our analyses of the RSs indicate there were only low concentrations of Cu–malate which explains this. The root elongation assay showed Cu–citrate was inactive in root elongation inhibition whereas the Cu-complexes with gluconate, malate, and especially DMA were active inhibitors. The citrate was likely correlated with toxicity because it was a response to that toxicity, whereas the gluconate and DMA complexes were likely active factors. DMA in particular is associated with solubilization of iron in iron-deficient environments, such as in high-pH calcareous soils, but it binds Cu as effectively as Fe,[59] which may explain why Cu reduced Fe nutrition in durum wheat.[39] Consequently, geochemical modeling showed that 100% of DMA was always complexed to Cu when present in all RSs where it could cause significant toxicity. Thus, we speculate that citrate but not malate or DMA exudation is protective against Cu toxicity in the root zone. Thus, our findings support that the biotic ligand model should be modified to include bioavailable metallo-organic complexes.

Conclusions

The addition of SPWs from calcareous soils in a sand growth matrix decreased the toxicity of CuO NPs to wheat, as seen by partial alleviation of the impact on root elongation and shoot K caused by the NPs. This information is relevant to bridge the gap between laboratory studies and field studies. Principal components analysis found root length in the sand systems to be negatively associated with free and dissolved Cu, suggesting bioavailability of both forms. Bioavailability of Cu, or other metals, could be used strategically in pesticidal or nutritional roles, both for the plant health and human consumption. On the other hand, metal NPs may have unintended detrimental effects in the rhizosphere. A bacterium, *Pc*O6, greatly altered the plant response to CuO NPs. The great variety of soil bacteria should be expected to have similarly large effects on plant response to metallic NPs.

These findings suggest that the phytotoxicity of CuO NPs will be altered in soils largely due to the chemistry of the soil fulvic acids and by the effects of root-associated microbes. More research is required to fully understand the connections between DNOM, root exudates, microbial products, and susceptibility to Cu toxicity. These findings also showed that the basis of the biotic ligand model in considering toxicity to be linked only to Cu ions is incomplete, because complexed Cu was also active. This study elucidates the complexity of processes occurring in the soil solution. Soil minerals and solid organic matter sorb ions in whole soils, which will additionally alter bioavailability and plant and microbial response from CuO NPs.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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