Interactions Between a Plant Probiotic and Nanoparticles on Plant Responses Related to Drought Tolerance

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Abstract

Colonization of certain probiotic microbes as a biofilm on plant roots induces beneficial responses that boost plant health. The surface colonization, biofilm formation, and production of plant-beneficial metabolites by probiotics on plant roots may be influenced by agricultural formulations containing nanoparticles applied as pesticides or fertilizers. A model system of wheat grown in sand is used to study seedling responses to CuO and ZnO NPs, applied at concentrations 300 and 500 mg metal/kg growth medium respectively. These NPs did not impair formation of layered biofilms on wheat seedling roots by Pseudomonas chlororaphis O6 (PcO6), a probiotic inducing drought tolerance. Plant growth with 300 mg/kg CuO NPs alone lowered shoot water content by 12% and changed the mechanical properties of the tissue compared with control plants. After a 6-d drought, shoots of 13-d seedlings were visibly more erect when seedlings were grown with Cu from NPs or ions than plants grown without Cu amendment. Growth of PcO6-colonized plants with CuO NPs induced lignification of the sclerenchyma in shoots, as well as increased nitric oxide (NO) accumulations in the wheat root, a metabolite associated with cell signaling in drought tolerance. These studies suggest that formulations containing selected NPs may interact positively with plant probiotics in promoting robust plant tissues and drought tolerance.

Keywords: biofilm, root morphology, lignification, shoot rigidity, nitric oxide

Introduction

Drought is a major stress with an impact on crop yield and plant growth.[1,2] Unpredictable rainfall and declining quality and quantity of irrigation water contribute to this global problem. Breeding for drought tolerance has variable success dependent on the crop.[3,4] Another potential strategy is to utilize plant microbiome isolates that induce drought tolerance.[1,2] Drought tolerance in plants is correlated with changes regulated in part by abscisic acid (ABA), reactive oxygen species (ROS) and nitric oxide (NO) signaling.[5–7] Increased expression of genes encoding protective proteins as well as those involved with enhanced levels of osmolytes, also aid plants in combating drought stress.[5–9]

Emerging strategies for sustainable agriculture include formulations employing nanoparticles (NPs). CuO and ZnO NPs are being considered as fertilizers, to supply essential elements, or at higher doses, pesticides.[10–13] Cu- and Zn-containing NPs also confer protection against drought stress in different plants.[13–17] Although Cu from CuO NPs enhances root hair formation, and Zn, from ZnO NPs, increases lateral root production,[18–21] the shorter root lengths perhaps reduce access to water. Enhanced wall lignification, as reported for *Arabidopsis*[18] and mustard[22] grown with CuO could alter water flow, and limit cell wall extension. Increased lignification is a documented response to plant drought stress.[23,24] Association of Cu ions with cell wall pectins may also impair water flow.[25,26] Increased anthocyanin for plants exposed to CuO NPs,[22] is consistent with water stress,[27] and elevated proline is a documented drought tolerance strategy.[28] These findings support that growth with CuO NPs establishes water stress that triggers drought protection measures. Wheat roots grown with CuO NPs exhibit enhanced accumulation of reactive oxygen species (ROS)[20] in agreement with the concept that plants meet NP challenges with a ROS burst.[29] Elevated ROS, a proposed consequence of increased ABA caused by drought stress, may signal transcriptional changes leading to stress tolerance.[6,7]

Drought tolerance also occurs from the association of plants with specific microbes.[1,2,15,30] Wheat root colonization with *Paenibacillus* and *Bacillus* sp. or *Pseudomonas chlororaphis* O6 (*Pc*O6) boosts plant survival under drought stress.[31,32] Drought stress for wheat with *Pc*O6-colonized roots show increased transcription for genes encoding products related to drought tolerance.[32] Few genes change in expression in a nonstressed plant, rather colonization with *Pc*O6 primes the plant to activate stress-tolerance genes with added stress by growth with CuO or ZnO NPs.[15] The microbial traits imparting drought tolerance are multifaceted and changes in root morphology are similar to those caused by NPs.[33,34] A drought-protectant *Paenilbacillus polymyxa* isolate stimulates root hair growth and lateral branching. [35] Remodeling allows greater access to rhizosphere water as well as providing more surface area for microbial colonization. Additionally, plant responses to such microbial

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metabolites as butanediol may be involved in induction to drought tolerance.[36] Butanediol reduces stomatal apertures and activates expression of the plant's stress-protective genes.[37] Butanediol and other metabolites from *Pc*O6 are produced as the cells grow as a biofilm on the root; the biofilm acts as a water-holding gel on root surface.[38,39] Greater drought tolerance in wheat is observed with a mutant of P. *polymyxa* producing enhanced biofilms than with the parental strain.[35] Biofilm formation is an essential trait for root colonization by ''biocontrol'' bacteria that protect the plant against microbialpathogens.[38–41]

The studies in this paper focus on processes connected with drought tolerance in plants. In *Pc*O6-colonized wheat seedlings grown with CuO NPs, shoots remain erect and water content is maintained for longer during drought.[15] Thus, we examined whether wheat shoots from noncolonized plants grown with CuO NPs had altered water content. CuO and ZnO NPs alter *Pc*O6 biofilm density on a root mimetic surface[42] and ZnO NPs impede biofilm formation by *Pseudomonas aeruginosa*.[43] Consequently, scanning electron microscopy (SEM) was used to characterize the *Pc*O6 biofilms on the root tips of control and NP-exposed wheat to see if formation was impaired by the NPs. Microbially-induced rapid lignification is a major process involved in induced systemic protection against pathogen challenge.[44] Because colonization by certain beneficial microbe also is reported to decrease lignification,[33] we examined whether the pattern of lignification was altered by growth with CuO NPs. Altered lignification could be involved in the rigidity of the erect shoots observed with drought-stressed Cu-exposed, *Pc*O6-colonized seedlings.[15,22] We also determined whether NO production was modified in *Pc*O6-colonized wheat roots when grown with CuO NPs. This finding would unite regulation drought protection and lignification observed in the *Pc*O6-colonized wheat planted under challenge with NPs. Findings of changes in lignification and strength of tissues induced by CuO NPs could be of significance in agriculture in growth of more robust crops.

Materials and Methods

NANOPARTICLES AND CHARACTERIZATION

Atomic force microscopy confirmed the nanodimensions of CuO and ZnO NPs purchased from Sigma-Aldrich, (St. Louis, MO) as single particles.[45,46] These ''as made'' particles were stored dry and in darkness, and were extensively characterized chemically (composition and crystallinity) and physically (size, shape). Various studies show their dissolution and aggregation under different environmental conditions.[45–48] Relevant methods and findings are shown in *Supplementary Table S1* (Supplementary Data are available online at www.liebertpub.com/ind).

PLANT GROWTH WITH AND WITHOUT DROUGHT STRESS AND NP EXPOSURE

Sterilized sand (300 g) in Magenta boxes was amended with dry NPs at defined doses between 10 to 300 mg metal/kg sand CuO NPs and 500 mg metal/kg sand for ZnO NPs depending on the study. No NPs were added to the boxes for control plants. These NP concentrations caused root morphological changes.[20] The sand was wetted with 50 mL sterile water prior to planting with twenty-five wheat seeds (*T. aestivum*, cultivar Dolores) in each box. The seeds had been surface sterilized in 10% hydrogen peroxide for 10 min and washed extensively with sterile distilled water. Control seeds were planted directly. Other seeds were planted after inoculation by submersion into a suspension of *Pc*O6 cells (1×10^5 cfu/mL) for 10 min. Each treatment was run in triplicate. Seedlings were grown at 28°C for 6 d with daily random rotation of boxes in a growth chamber. Colonization of plant roots by *Pc*O6 was confirmed by plating root segments directly onto Luria Broth medium plates and observations of the growth of bright orange colonies indicative of this bacterium around the root surface.[21]

To determine whether water content of shoots was affected by CuO NPs, shoots were harvested from seedlings grown with the NPs but without *Pc*O6-colonization and weighed immediately to obtain the wet weight. Weight was measured after drying at 60°C for 48 h and water content of fresh tissues determined.[49]

In other studies to probe the role of metal ions on shoot rigidity, the NPs were replaced with sterile solutions of $CuCl_2$ or $ZnCl_2$ (20 mg metal/L). The 6 d-old seedlings were drought stressed for 6 d by removing the lids of the growth boxes and withholding further water.

SEM/EDS ANALYSIS OF PLANT ROOTS

Root tips (0.5 cm) were excised from washed wheat roots and immersed into 100% methanol for fixation. The roots were dehydrated with ethanol and chemically dried in solutions of hexamethyldisilazane following the methods previously reported.[50,51] The dried root tips were mounted to aluminum stubs with carbon tape and coated with a 10 nm layer of Au 60%/Pd 40%. The samples were imaged by SEM (FEI Quanta FEG 650). Chemical elements at specific points on the surface were obtained using energy dispersive X-ray spectrometry (EDS) (Oxford). Images and spectra were collected under high vacuum (10^{-6} torr) at an electron energy of 20 keV.

MECHANICAL PROPERTIES, NITRIC OXIDE PRODUCTION AND LIGNIFICATION OF TISSUES

Shoots of 7 d seedlings, raised with and without 300 mg Cu/kg from CuO NPs, were individually cut and sectioned into 30 mm lengths, followed by immediate testing to minimize water loss by transpiration. Shoot cross-sections were measured using digital calipers to allow force to be converted to engineering stress using the original cross-sectional area. The cut ends of the shoot sections were capped with adhesive tape to facilitate mounting in the grips of a mechanical testing frame (Instron Model 5542 with Bluehill Operating System, Norwood, MA) equipped with a 50 N load cell. The Instron is routinely used to examine the mechanical properties of plant tissues.[52,53] The functionality of the machine was checked for accuracy and reproducibility between experiments using standard dog-bone polydimethylsiloxane samples, as recommended by the American Society for Testing and Materials. The shoot sections were extended at a rate of 3 mm/min and load and extension were recorded until failure. From the load versus extension data, values for stress, strain, tensile strength, and toughness were calculated for each sample using the software from the system. Seven independent studies were run with between 11 and 22

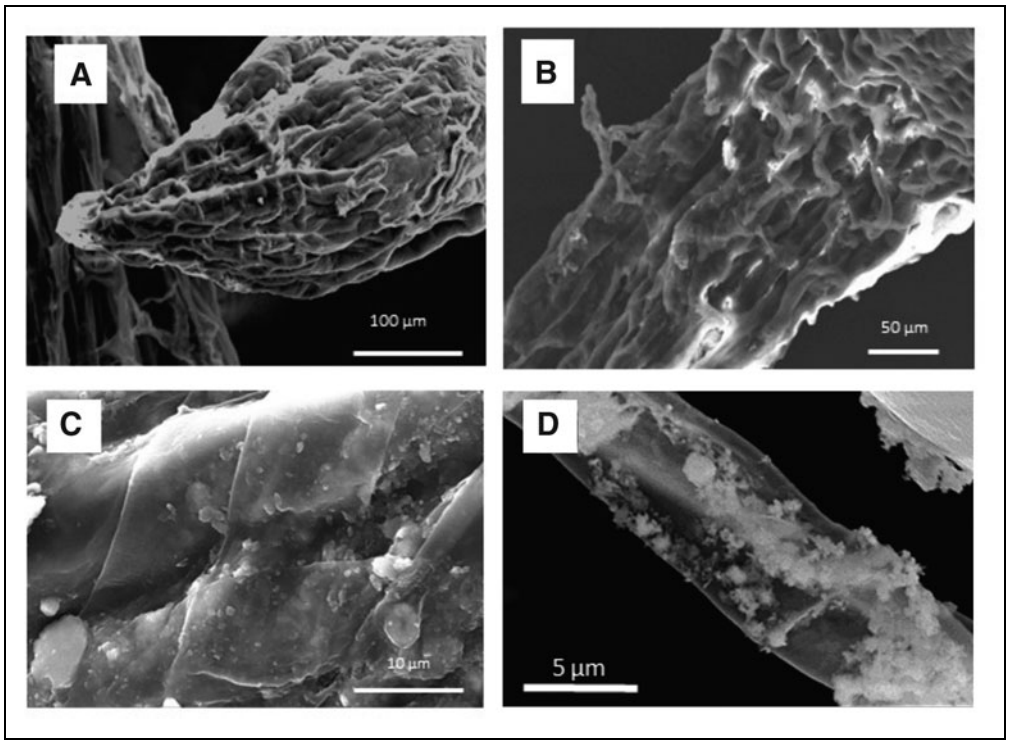

Fig. 1. Root tips of control seedlings grown without *Pc*O6 colonization or NPs: **(A)** root cap; **(B)** transition zone of division; **(C)** collapsed root hairs; **(D)** image of a root hair from a seedling grown with 300 mg Cu/kg from CuO NPs but without *Pc*O6 colonization. All images were collected with a FT detector at 10^4 Torr, 20,00 KV, 2.0 spot size and 15 μs dwell time.

shoots being sampled used in each study for totals of 121 shoots from control plants and 108 shoots from plants grown with CuO NPs. For statistical analysis, a one-way ANOVA was run using JMP8 software to determine P values for differences in mechanical properties for growth with and without CuO NPs using the combined data for all studies.

NO production in the root cells was indicated by using 4-amino-5-methylamino-20,70-difluorofluorescein diacetate (DAF-FM DA) (Life Technologies, Carlsbad CA).[54] The root tips were treated for 30 min with 5 mM dye from a solution in dimethyl sulfoxide (DMSO; Life Technologies) and rinsed by immersion for 15 min in sterile distilled water. Excitation at 488 nm and emission at 566 nm was used to reveal fluorescence. When tips were treated with DMSO without DAF-FM DA, water washed and examined; no autofluorescence was observed. Each treatment was examined using three root tips and at least three independent studies were performed. The images were processed uniformly to enhance brightness and contrast.

To assess lignification, root tips (0.5 cm) and hand-cut sections of shoots were immersed in phloroglucinol-HCl and imaged under a light microscope.[22] Red coloration indicated lignification of the plant cell walls. Data shown are typical results obtained from three independent studies, each with three root tips being examined from three separate plants per treatment.

Results

PCO6 BIOFILM FORMATION AT ROOT SURFACE

SEM images of control root tips grown without NPs and without *Pc*O6 colonization showed clear surface features including collapsed root hairs, defined particles, and typical brick- shaped cells at the root cap (*Fig. 1A-C*). No surface mucilage was observed on the root surface. Imaging of the root hair zone of seedlings grown without *Pc*O6 colonization but with CuO NPs also lacked mucilage (*Fig. 1D*). In contrast, SEM imaging revealed biofilms overlying the root surface in the regions of the root hairs when the plants were grown from *Pc*O6-inoculated seed (*Fig. 2*). This material had variable morphology ranging from waxy-looking scales to solid-appearing agglomerates to a lacey lattice (*Fig. 2*). The biofilm was thick, layered and complex in the root hair regions, where it appeared to act like a glue to embed particles. The biofilm prevented visualization of root hairs in the SEM samples (*Fig. 2*). EDS analysis of particles in the *Pc*O6-biofilm webs showed CuO NP- grown

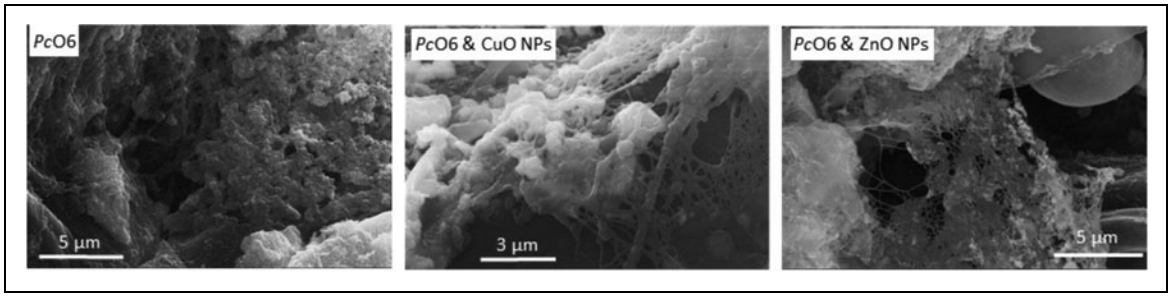

Fig. 2. Images from the root hair zones of roots colonized by *Pc*O6 and grown with or without 300 mgCu/kg from CuO or 500 mg Zn/kg from ZnO NPs.

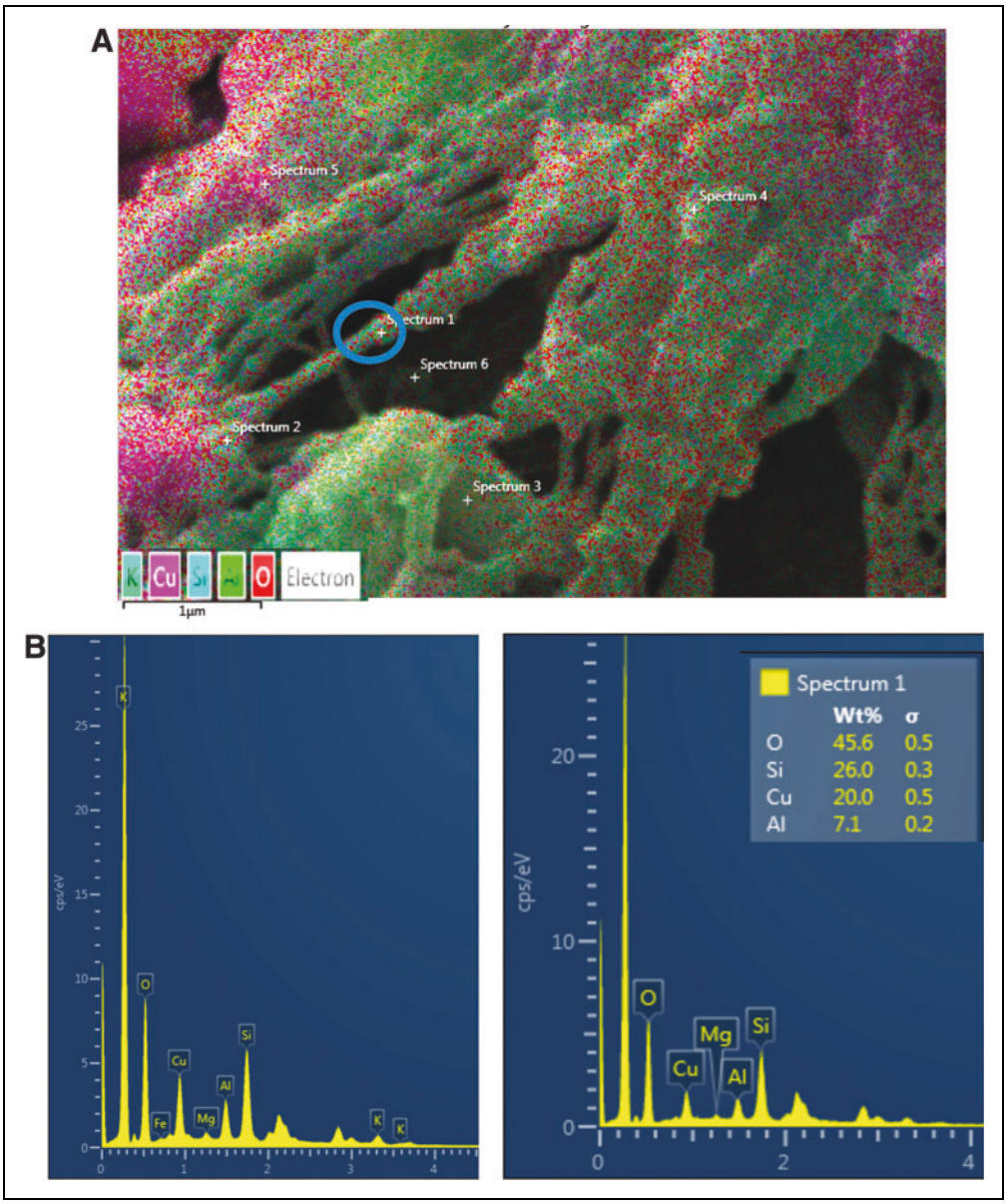

Fig. 3. **(A)** Image of a biofilm from root hair zone of seedlings grown with *Pc*O6 and 300 mg Cu/kg from CuO NPs overlaid by EDS spectra with the different major elements being shown by different colors. The blue circle shows the region in a thread of the biofilm where a point analysis was taken (Spectrum 1). **(B)** The spectral map and EDS spectrum at site 1. Color images available online at www.liebertpub.com/ind

roots had a Cu signature but no detectable Zn (*Fig. 3A,B*). Conversely, EDS of the biofilms on ZnO NP-grown roots in the root hair zone showed particulates with a Zn signature but no detectable Cu signals (*Fig. 3*). The root caps of seedlings grown with *Pc*O6 colonization lacked biofilms (*Fig. 1*, *Supplementary Figs. S1A* and *S2)* for colonized seedlings grown with CuO NPs and ZnO NPs, but showed particles. EDS analysis of particles on the root cap from *Pc*O6-colonized plants grown with CuO NPs had high Cu signals. Other spectra were dominated by Si, likely indicating that these particles were from the sand. (*Supplementary Fig. S1A,B*). The image in *Supplementary Fig. S2* illustrates root caps from *Pc*O6-colonized plants grown with ZnO NPs where EDS analysis of clearly visible particles showed strong Zn signals but no Cu signals. The image in *Supplementary Fig. S3* is from the root hair zone of ZnO NP-grown seedlings. Here, biofilm threads are visible as well as bacterial cells that are attaching to the surface through columns of mucilage. EDS analysis at one point of the stippled surface shows a strong Zn signal. Although examined over many images, no discrete outlines indicative of bacterial cells were seen with the biofilms on the roots of seedlings grown with CuO NPs.

WATER STRESS, LIGNIN AND NITRIC OXIDE PRODUCTION

The shoots of *Pc*O6-colonized seedlings grown with 300 mg Cu/kg CuO NPs and drought stress had higher water content and appeared to be more erect than those of control plants.[15] Thus, increased turgidity is thought to account for reduced wilt in the *Pc*O6-colonized plants.[15] However, erect shoot growth also was observed (*Fig. 4A*) for seedlings grown without *Pc*O6 colonization but with exposure to CuO NPs after the drought period. Growth of the noncolonized plants with CuO NPs showed dose-dependent reduction in the water content of the shoots (*Fig. 4B*); at 300 mg Cu/kg the shoot tissues had 12% less water than tissues of plants grown without Cu amendment. Consequently, the erect growth habit could not be attributed to turgidity. It seems likely that Cu released from the CuO NPs was involved in causing the erect shoot growth because shoots of noncolonized seedlings grown with Cu ions also were more erect than the controls after the drought stress period (*Supplementary Fig. S4*). The effect was specific to Cu because erect shoots were not observed from wheat grown with Zn ions upon drought stress (*Supplementary Fig. S4*).

Growth of colonized plants with 10 or 300 mg Cu/kg from NPs revealed changes in the deposition of lignin compared to the tissues from control seedlings. For colonized seedlings grown with CuO NPs, intact roots (*Supplementary Fig. S5*) displayed zones of red coloration surrounding the stele when

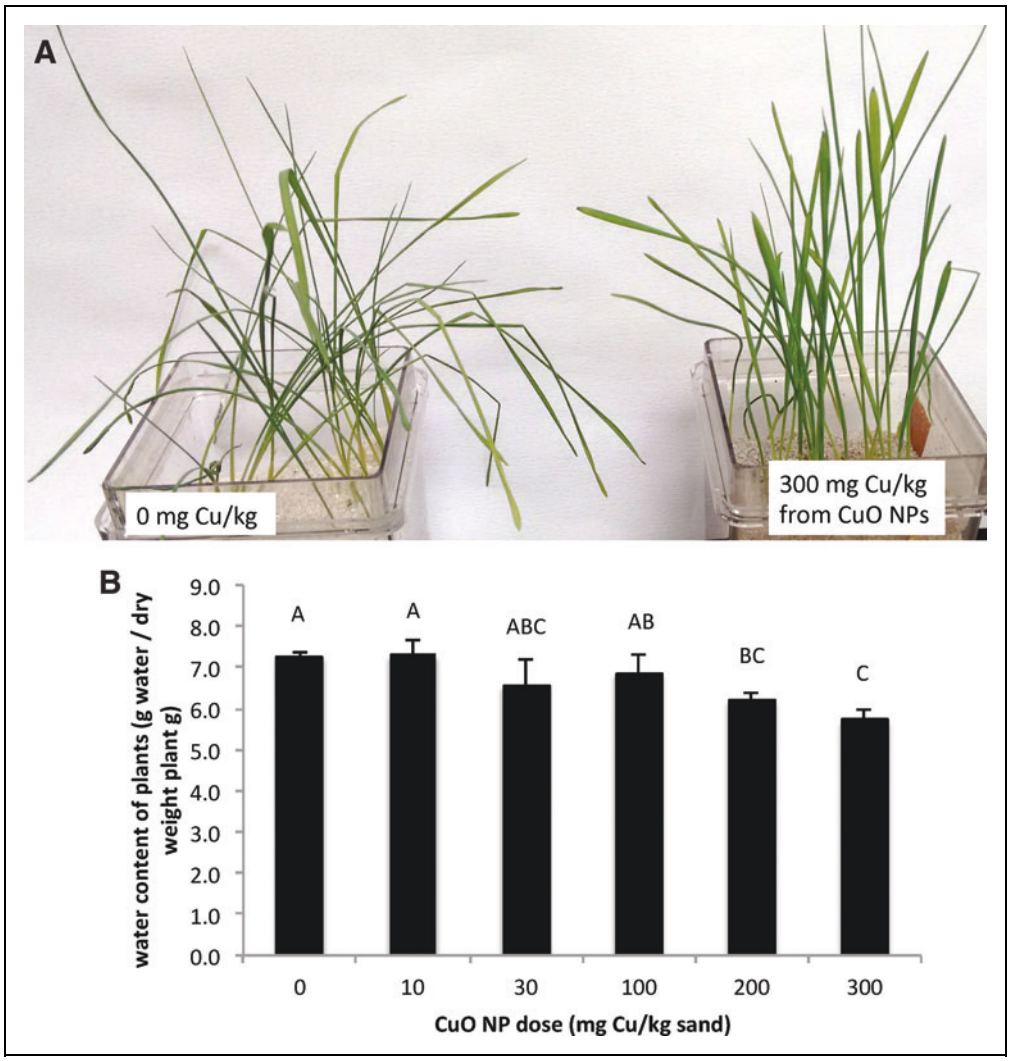

Fig. 4. Growth of noncolonized wheat with CuO NPs causes shoot tissue to be more erect than tissues grown without these NPs after a drought period. The plants shown were grown for 6 d with normal hydration and droughted for 6 d, a process which accentuates the visible stiffness of the CuO NP-grown plants. **(B)** Effect of growth of wheat seedlings with CuO NPs on the water content of shoots. Data are expressed as g water/g dry shoot. Data show the means of three separate studies for each treatment. Columns with the same letters are not significantly different ($\alpha = 0.05$) by Tukey's HSD. Error bars represent 95% CI. Color images available online at www.liebertpub.com/ind

treated with phloroglucinol. For the shoots of the colonized seedlings grown with CuO NPs, not only were cells in the vascular bundles stained intensely for lignin, as also observed for shoots from control seedlings, but a novel observation was the intense red staining apparent in the bundles of sclerenchyma cells (*Fig. 5*).

Increases in green fluorescence indicative of NO accumulation were apparent for roots colonized with *Pc*O6, with and without growth with CuO NPs, after treatment with DAF-FM-DA (*Fig. 6*). The images for sections from the colonized and CuO NPs-grown roots (*Fig. 6*) appeared obscured, likely due to the proliferation of elongated root hairs covering the root surface.[20] An increase in NO accumulation by exposure to Cu was confirmed by observation of bright green fluorescence in roots of noncolonized seedlings grown with an amendment of Cu ions (*Fig. S6*).

EFFECT OF CUO NPS ON MECHANICAL PROPERTIES

To understand whether growth with CuO NPs changed the mechanical properties of the shoots causing greater stiffness, extensibility studies were run using Instron instrumentation.[52,53] The stress versus strain curves (see *Supplementary Fig. 7* for examples) resembled those published for barley tissues with a linear response that ended in a break point as the tissue fractured.[53] Toughness, which is the area under a stress vs. strain curve, reflects the ability of a sample to absorb energy imparted during mechanical loading. This property is highly relevant to the deformation observed in drought stressed shoots, which is observed visually as wilt. It is also relevant to lodging due to wind, or other physical damage.[55] Toughness and tensile strength were significantly higher based on analysis with a one way ANOVA for treatment ($P \leq 0.001$) and statistical differences were confirmed with Students T test analysis. The values for toughness, 156 J/m^3 for control shoots versus 183 J/m^3 for the shoots grown with CuO NPs, showed a 17% increase. A 12% increase was seen for the tensile strength with values for the control shoots of 7.5 MPa and 8.4 MPa for the shoots from seedlings grown with CuO NPs. Colonization of roots with *Pc*O6 had no significant effect on these mechanical properties measured using the Instron instrument under the conditions used for assay (data not shown).

Discussion

The formation of layered biofilms of complex architecture by a plant-probiotic bacterium, *Pc*O6, on wheat seedling roots was not impaired by growth with CuO or ZnO NPs at concentrations that changed root morphology. These findings were from a model growth system where sand was used as the growth matrix and only a single root-associated microbe was used. In field soil, soil texture would be complex and the organic materials would influence the bioactivity of the NPs. For simplicity, also only a single root-colonizing microbe, *Pc*O6, was used, whereas the

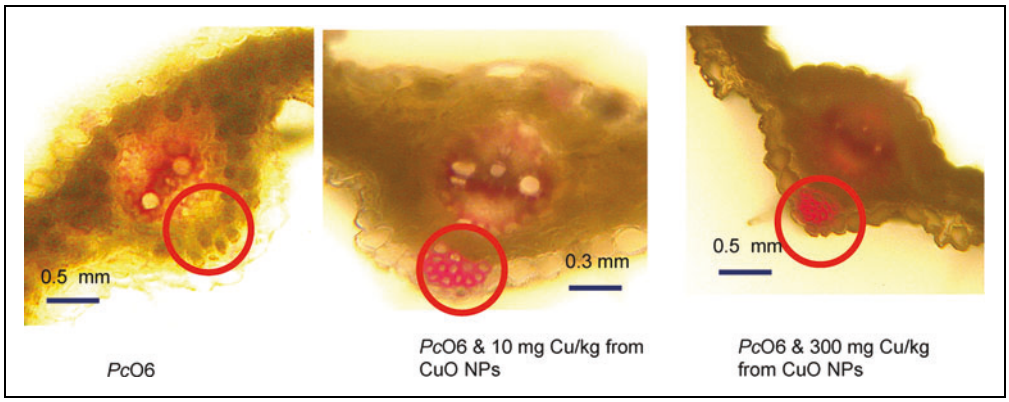

Fig. 5. Images of transverse hand-cut sections of shoots from *Pc*O6-colonized plants grown 7 d with and without CuO NPs after treatment with phloroglucinol-HCl. The circles show the sclerenchyma cells which are stained red due to lignification when the leaf was from seedlings grown with CuO NPs. The images are typical of leaf sections from three leaves harvested from different seedlings and two different studies. Color images available online at www.liebertpub.com/ind

plant's microbiome in a productive agricultural soil would have many members. However, this model system gave consistency in measurements and observations. SEM imaging of the root tissues for the presence of the biofilm confirmed previous studies where colonization was assessed through determining the culturable *Pc*O6 cells released from plant roots.[21] The *Pc*O6 biofilms were prolific at the root hair zones, presumably because the bacterial cells were using the metabolites released by the plant as nutrients.[21] Root hairs actively transport metabolites out, and nutrients and water into the plant.[56,57] We speculate that the absence of mature biofilms at the root cap may be explained by frequent sloughing of these specialized cells.[20,58] The lack of biofilms at the zones of elongation and division may reflect on there being insufficient time for the bacteria to produce a mature biofilm matrix. Other root surface images have shown clusters of *Pc*O6 cells overlain with a sparse lacey layer[59] or without an overlay but attached by small projections.[42] We speculate that these clusters were sites where biofilm formation was just being initiated. The array of different physical structures in a mature biofilm raises the question of variability in composition and in quantity of the polymers in the biofilm matrix. We suggest that the inability to see individual cells in the biofilms produced with CuO NPs could be related to greater gel formation supported by the NP-increases in malate or citrate documented to be released by the roots.[49] Enhanced production of extracellular polymers that masked the outlines of bacterial cells was observed for *Pc*O6 biofilms on a nonbiological surface with increases of citrate in the growth medium.[42]

The process of biofilm formation was not inhibited by growth with the NPs, even though toxic effects of both Zn and Cu could be expected.[45,60] At sublethal levels different aspects of the metabolism of *Pc*O6 are altered by CuO and ZnO NPs, as summarized in *Supplementary Table S2*. The *Pc*O6 biofilms on the root surface showed loading of Cu or Zn when plants were grown with the NPs and increased shoot metal loads relative to those in control plants have been observed.[15,21] Loading of metal from nano-Zn into grains is reported by other groups such as for sorghum.[61]

The *Pc*O6 cells at the root surface were metabolically active, producing the gel matrix of the biofilms and other metabolites. For example, *Pc*O6 cells colonizing bean roots exposed to ZnO NPs, secreted a fluorescent pyoverdine-like siderophore[46], agreeing with the observation that these NPs increased siderophore production in planktonic *Pc*O6.[62] The observations of strong *Pc*O6 biofilms on the root surface, even in the presence of CuO or ZnO NPs, were correlated with greater tolerance to drought stress.[15] Thus, *Pc*O6-colonization involving biofilms

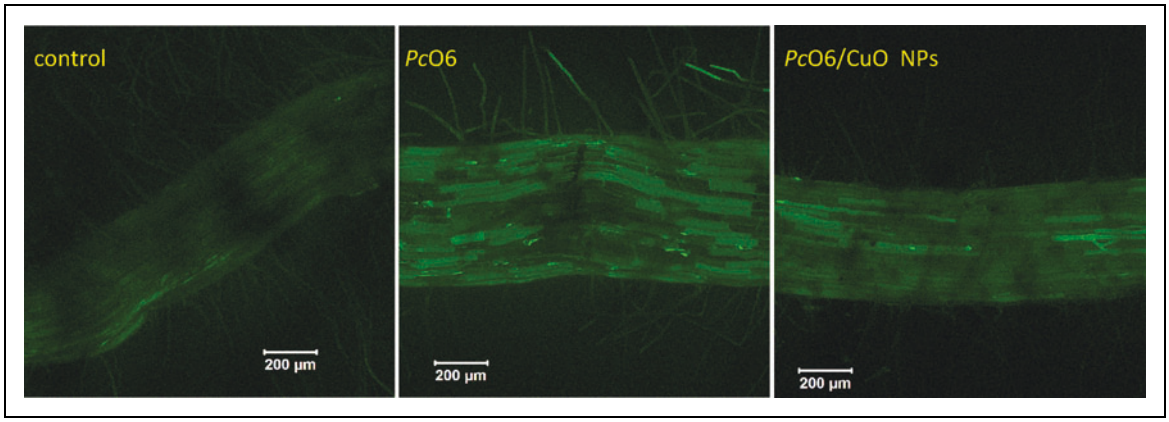

Fig. 6. Images of NO accumulations in root tissues at the root hair zones in seedlings grown with and without *Pc*O6 colonization, and growth with CuO NPs. The root segments were stained with DAF-FM DA to reveal NO accumulation by procedures described herein. Color images available online at www.liebertpub.com/ind

appeared to counteract the effect of reduced shoot water when noncolonized wheat seedlings were grown with CuO NPs. These findings further support the importance of biofilm formation as a trait for beneficial plant microbes. Studies are in progress to characterize the polymers crucial in the matrix of the *Pc*O6 biofilm for attachment and the three-dimensional complexity of the structures, especially as they relate to water withholding and metal binding. The findings suggest agricultural formulations of NPs could be tuned to boost the potential of the beneficial microbes such as in induction of drought tolerance.

Drought stress tolerance in *Pc*O6-colonized wheat roots occurred in seedlings that displayed changed patterns of lignification in root and shoot tissues upon growth with CuO NPs. The relationship between lignification, drought and growth is complex. Lignification is a proposed symptom of drought that leads to reduced growth.[22,23,63] However, additionally lignification could be a strategy to limit Cu phytotoxicity, based on similar findings of plants exposed to Cd.[64] Cu modification to plant cell walls through lignification and the association of Cu with pectic fractions[26] could be involved in reduced apoplastic water flow, accounting for lower shoot water content, that would prime the plant for tolerance to drought. Indeed, a recent paper documents that growth with CuO NPs caused increased both lignin and pectin components of the plant cell walls.[65] Drought stress protection may be correlated with superoxide anion signaling in *Pc*O6-colonized roots grown with CuO NPs.[21] The results from this paper find that elevated Cu and *Pc*O6 also enhanced NO formation, which has been implicated as a signal for increased lignification, the induction of drought protective responses and Cu tolerance.[66–68] Consequently, we see overlap in potential signaling responses of wheat to confer drought stress tolerance imposed by growth of *Pc*O6-colonized seedlings with CuO NPs.

A novel finding from these studies is the Cu-induced increased lignification in the sclerenchyma cells of the shoots. This augments the recent findings using spectral methods for the whole shoot tissue of enhanced lignification in shoots by CuO NPs.[65] Lignification of sclerenchyma is associated with more robust shoots, which display higher resistance to wind- caused lodging.[55] Growth with CuO NPs increased toughness and tensile strength of the shoots, findings that could be explained by lignification of shoot sclerenchyma cells. Cell wall strengthening by lignification also is correlated with improved resistance to invasion by microbial pathogens.[44] Consequently, Cu-containing NP formulations, able to stimulate lignification, could additionally boost plant robustness and pathogen resilience.

The ability of NP formulations, especially those containing Cu, Zn and Ag, to combat pathogen challenge of plants is becoming well researched.[69] A recent paper illustrates control of disease in maize with applications of Cu-chitosan NPs.[70] These NPs have double action, through Cu release that could have toxic impact on the microbial pathogens directly as well as through the plants response to chitosan, an activation of disease resistance.[71] Of interest is that chitosan protection involves increased levels of abscisic acid that could regulate expression of drought tolerance genes in the plant.[71,72] Thus, the nanoformulations may activate multiple protective pathways. In the field, these processes may occur along with synergism with responses due to root colonization with a beneficial microbe, such as the isolate *Pc*O6 used in these studies. These effects may teem with the fertilizer effects of nanoformulations of Cu and Zn that enhance the levels of these essential metals.[61] However, the doses of nano-Cu or nano Zn products would need to be gaged to avoid potential decreases in root growth and, for CuO NPs, reduced shoot water content. For instance, growth in acidic soils, promoted dissolution of CuO NPs causing higher phytotoxicity than growth in calcareous soils suggesting that with low pH pore waters low doses would be sufficient.[73,74] Water management in the plant, reduced by the NPs, could be balanced by the presence of a probiotic, such as *Pc*O6 that primes plant tissues for stress tolerance.[1,2,31,35,38]

The highlights of the findings are summarized in the illustration in *Fig. 7*. Overall the results suggest that the use of CuO NPs in agricultural formulations could be tuned to boost plant performance. The responses induced by CuO NPs, which include enhanced lignification, improved physical robustness and nutrition of the tissues, and synergism with probiotics for increased

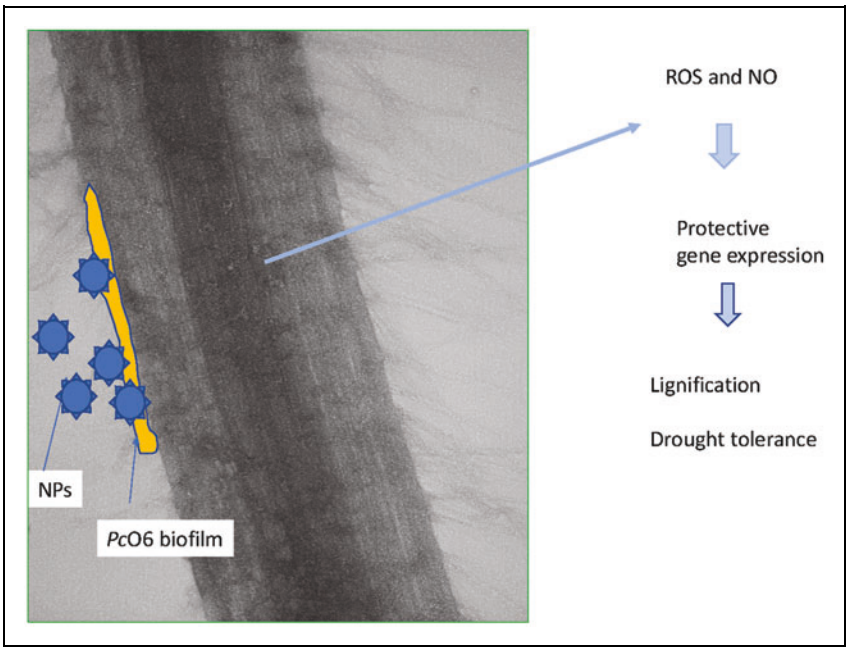

Fig. 7. CuO nanoparticles (NPs), and/or their released ions, at the wheat root surface interact directly with plant root cells or with patchy biofilms with complex morphology of Pseudomonas chlororaphis O6 (PcO6). The interactions with the bacterium in the presence of the NPs change plant metabolism resulting in drought tolerance. In this paper, we show accumulations of nitric oxide (NO) at the root surface, which could act with other signals, such as reactive oxygen species (ROS), to trigger changes in gene expression for protective responses to drought. The observations of increased lignin deposition in support tissues, such as the sclerenchyma in shoots, and drought tolerance could be valuable in field-grown plants. Color images available online at www.liebertpub.com/ind

tolerance of plants to environmental stresses, could be valuable in field-grown plants. The findings also indicate that the formation of biofilms by probiotic microbes on the root surface should be an additional trait monitored in studies to improve plant performance under stress that will occur in agricultural settings.

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Authors Disclosure Statement

No competing financial interests exist.

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