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Remodeling of root morphology by CuO and ZnO nanoparticles: effects on drought tolerance for plants colonized by a beneficial pseudomonad.

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Abstract Formulations that include nanosized-CuO and -ZnO are being considered for agricultural applications as fertilizers because they act as sources of Cu or Zn. Currently, few studies of the effects of these nanoparticles (NPs) consider the three-way interactions of NPs with the plant plus its microbiome. At doses that produced root shortening by both nanoparticles (NPs), CuO NPs induced proliferation of elongated root hairs close to the root tip and ZnO NPs increased lateral root formation in wheat seedlings (*Triticum aestivum*). These responses occurred with roots colonized by a beneficial bacterium, *Pseudomonas chlororaphis* O6 (*Pc*O6), originally isolated from roots of wheat grown under dryland farming in calcareous soils. The *Pc*O6- induced tolerance to drought stress in wheat seedlings was not impaired by the NPs. Rather growth of the *Pc*O6-colonized plants with NPs resulted in systemic increases in expression of genes associated with tolerance to water stress. Increased expression in shoots of other genes related to metal stress was consistent with higher Cu and Zn levels in *Pc*O6-colonized shoots grown with the NPs. The work illustrates that the plants grown with CuO or ZnO NPs showed cross protection for different challenges of metal stress and drought.

Key words Beneficial bacterium; Drought stress response; Nanoparticles; Root colonization.

Introduction

Food security is a major factor addressing the world where population size, climate variability, urbanization of land and water quality and supply are problematic. Drought is one of the most significant threats that influence plant yield and quality. Strategies that reduce the negative effects of drought are under investigation (Mwadzingeni et al. 2016; Nezhadahmadi et al. 2016) and could include better utilization of microbial rhizosphere colonists that improve plant growth under adverse conditions (Pieterse et al. 2014; Martinez-Medina et al. 2016; Timmusk et al. 2017).

The bacterium, *Pseudomonas chlororaphis* O6 (*Pc*O6), is an example of a beneficial rhizosphere microbe that improves plant health. This isolate was cultured from the roots of wheat at the end of the growing season in calcareous soil under dry land farming conditions, which involved periods of drought (Spencer et al. 2003). Laboratory studies show induced tolerance to drought in *Pc*O6-colonized *Arabidopsis* correlates with enhanced transcription from genes associated with protection from water stress (Cho et al. 2013). Also, higher levels of an osmolyte, galactinol, are associated with drought stress tolerance observed in cucumber with roots colonized with this bacterium (Kim et al. 2008). This paper questions whether induced drought stress tolerance is affected by plant growth with concentrations of nanoparticles (NPs) that cause change in root morphology.

Growth of plants with CuO and ZnO NPs shows dose-dependent shortening of roots (Lin and Xing 2008; López-Moreno et al. 2010; Nair and Chung 2015; Deng et al. 2016). In wheat, the CuO NPs additionally trigger proliferation of elongated root hairs close to the root cap, responses that are duplicated by Cu ion treatments or exposure to

exogenous indole acetic acid (IAA) (Adams et al. 2017). Vitti et al. (2014) similarly report increased root hair density for Cu-exposed *Arabidopsis*. In contrast, the shortened roots resulting from growth with ZnO NPs have enhanced lateral root formation (Watson et al. 2015). Similar findings are reported for Zn ions for other plants, such as the grass, *Festuca rubra* (Powell et al. 1998). The same response of increases in lateral root formation occurs with the toxic metal Cd in *Arabidopsis* (Vitti et al. 2014). Both lateral roots and root hairs are associated with water and nutrient uptake into the plant (Gilroy and Jones 2000). Consequently, the main goal of the studies reported in this paper was to determine whether growth with NPs at doses that affected root morphology would affect tolerance to drought induced by *Pc*O6.

Our previous studies with plants grown with exposure to NPs at doses causing root shortening and root hair proliferation show an interactive effect between microbial root colonization and responses to NPs (Wright et al. 2016). Although wheat root colonization by *Pc*O6 did not change the expression of a selected array of genes in the root tissues, colonization reduced expression for genes induced by seedling growth with CuO NPs. These plant genes include those related to tolerance to heavy metal stress and reactive oxygen species (ROS) as well as genes regulated by abscisic acid (ABA), a signaling compound controlling plant responses to water stress (Wright et al. 2016). Because *Pc*O6 induces systemic effects in plants (Spencer et al. 2003), another goal of the studies in this paper was to examine transcripts in shoots to gain better understanding of the impact of NPs on *Pc*O6-induced drought tolerance.

The response of wheat to drought stress was examined morphologically and by determination of water retention in leaf tissue. These findings were compared with

differences in systemic gene expression seen in shoots for plants grown with *Pc*O6-colonized roots and with either CuO or ZnO NPs as determined by a wheat gene chip array. Changes in expression for genes selected because they had altered expression in the leaves were also examined by RT-PCR analysis. These findings are relevant to a better understanding of the potential use of NPs in agricultural products. NPs containing essential minerals such as Cu and Zn (Grotz and Guerinot 2006; Sinclair and Kramer 2012), are being considered in fertilizer formulations (DeRosa et al. 2010; Servin et al. 2015; Monreal et al. 2016; Wang et al. 2016).

Materials and methods

Nanoparticles and characterization

The nanoparticles of CuO, with nominal size 50 nm, and ZnO NPs, with nominal size 70 nm, were purchased from Sigma -Aldrich, Mo, USA. Atomic force microscopy confirmed the nanodimensions of single particles (Dimkpa et al. 2012). The particles were weighed “as is” and directly transferred dry into a sand growth matrix for the plant studies (Dimkpa et al. 2013). These particles have been extensively characterized chemically (composition and crystallinity) and physically (size, shape, dissolution and aggregation) as described in previous publications (Dimkpa et al. 2012, 2013, 2015).

Plant growth with and without drought stress

Wheat seeds (*T. aestivum*, cultivar Doloris) were surface sterilized in 10 % hydrogen peroxide for 10 min and washed extensively with sterile distilled water. Seeds were planted directly or after inoculation by submersion into a suspension of *Pc*O6 cells (1×10^5 cfu/ml) for 10 min. The seeds were grown in sterilized sand (300 g) wetted with 50 ml sterile water, with and without additions of NPs at 300 mg metal/kg sand for CuO

NPs and 500 mg metal/kg sand for ZnO NPs. Element analysis of the sand (Dimkpa et al. 2013) showed that it was not a source of Cu or Zn. Each treatment was run in triplicate. Twenty-five seeds were placed into each growth box. Seedlings were grown for 7 d with daily random rotation of boxes in a growth chamber at 28 ˚C.

To induce drought, lids of the boxes were removed at 7 d and air drying was allowed for 6 d at which time the control plants were beginning to wilt. The shoots were cut, weighed and lengths measured. Shoots were dried at 65 °C until no further change in weight, due to moisture loss, was observed. The water loss/seedling was determined. Leaves were stripped of their coleoptiles, digested with nitric acid and the extracts examined by ICP-MS to determine metal loading as described in Watson et al. (2015). Roots, harvested from the sand matrix after leaf excision, were rinsed in sterile water to remove sand grains before determining root length, and imaging to show morphology. *Pc*O6 colonization of the root surface was confirmed by plating intact roots onto Luria-Bertani (LB) agar. To quantify cell density, each root was transferred to 10 ml sterile water, vortexed for 1 min to release attached cells, and the resulting suspension serially diluted before plating onto LB agar plates. Colony forming units (CFU) were counted after 48 h incubation at 28 ˚C.

Analysis of systemic gene expression

Microarray analysis was performed with the Agilent wheat chip (G2519F) with a 4 x 44K format with 43,805 wheat probes to assess changes in transcripts triggered in the leaves of *Pc*O6-colonized plants by growth with the NPs The chip was based on open sources, including RefSeq,UniGene, TIGR Plant Transcript Assemblies, and TIGR

Gene Indices, with data available in 2008. Consequently, the probes on the chips were biased towards genes involved in grain development, responses to drought and to challenge by pathogens such as *Fusarium graminearum*.

Total RNA was extracted from the leaves of 7 d seedlings grown (1) without *Pc*O6 colonization and without NPs, (2) without NPs but with *Pc*O6 inoculation, (3) with CuO NPs plus *Pc*O6 colonization and (4) with ZnO NPs plus *Pc*O6 colonization. The extracts were prepared using the RNeasy Mini Kit (Qiagen) with liquid-nitrogen frozen leaves. Batches of RNA were produced from three individual growth studies and pooled. The RNA was used to prepare cDNA, as described in Wright et al. (2016), using AMV reverse transcriptase (Promega). The cDNA was used in microchip analysis performed by MOgene Dx, St Louis, MO, USA. Each of the pooled batches of RNA was processed on three different arrays. Only those responses that yielded at least log2 changes in gene expression for all three arrays were scored. The mean fold change was recorded for changes due to treatments 2, 3 or 4 against the control (treatment 1) mean value. Thus, the changes in gene expression in the leaves for treatments with *Pc*O6, *Pc*O6 plus CuO NPs or *Pc*O6 plus ZnO NPs were compared with expression in leaves from control plants grown without colonization or exposure to NPs. For selected genes, Q-PCR was run using the cDNA prepared from RNA extracted from leaves with the same 4 treatments, plus a treatment where leaves were harvested from seedlings grown without *Pc*O6 colonization but with CuO NPs. The PCR was conducted with SYBR Green detection in a Bio-Rad iQ5 real-time PCR detection system (Bio-Rad Laboratories) in 96-well plates (Thermo Scientific). Gene expression levels were

determined using the Delta-Delta-CT method with normalization to expression from the wheat gene encoding a *ADP*-ribosylation factor control (Wright et al. 2016).

SEM analysis of plant roots

Root sections were excised 0.3 cm from the visible root tips from washed wheat roots and immersed into 100% methanol for fixation. The root tips were dehydrated with ethanol and chemically dried in solutions of hexamethyldisilazane following the methods reported in Talbot and White (2014) and Kashi et al. (2014). 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 scanning electron microscopy (SEM) (FEI Quanta FEG 650).

Results

Drought stress response in *Pc*O6 plants grown with CuO and ZnO NPs

Control plants raised without exposure to NPs and without root colonization by *Pc*O6 showed wilt symptoms by 6 d of water withholding for seedlings grown for 7 d with normal hydration (Fig. 1). The droughted plants raised with *Pc*O6 colonization but without exposure to the NPs had shoots that were more erect than the control plants (Fig. 1). The *Pc*O6-colonized wheat seedlings, when grown with CuO NPs, had even more erect leaves after 6 d of drought than leaves of the control plants (Fig. 1). There was little difference in leaf rigidity between *Pc*O6-colonized plants and the colonized plants grown with ZnO NPs (Fig. 1).

Determination of shoot wet weight/seedling showed that all shoots from *Pc*O6-colonized plants had higher water contents than plants raised without the bacterium (Fig. 2). After drying, the water loss from leaves/seedling confirmed a higher water content from leaves of *Pc*O6-colonized plants than those from control plants (Fig. 2). These changes persisted in when colonized plants were grown with the NPs (Fig. 2).

Systemic effects of *Pc*O6 colonization and growth with NPs on gene transcription in shoots

Differential gene expression was observed in leaves of plants grown with *Pc*O6 colonization or *Pc*O6 colonization plus CuO or ZnO NPs with respect to the control plants that were grown without NPs or *Pc*O6 colonization. The microarray analysis showed that certain genes were uniquely activated or downregulated by a treatment whereas many others overlapped in gene regulation between treatments (Fig. 3). The complete microarray data are provided as Supplemental data. The range of activation and down regulation in gene expression was greatest for the treatment with CuO NPs plus *Pc*O6 (Supplemental Table 1). Changes in expression clustered mainly in the 2-4 - fold range (Supplemental Fig. 1).

Inspection of the microarray data showed genes encoding functions was associated with water stress (i.e., LEA proteins and dehydrins as well as aquaporins (Table 1). A subset of genes also encoded proteins involved in synthesis of different types of osmolytes, including carbohydrates, putrescine and betaines (Table 1). These osmolytes protect protein and membrane structures from water deficits and ROS stress.

Genes that condition metal homeostasis also showed upregulation in shoots grown with NP exposure (Table 2). These genes encoding proteins that act as

chaperones for metal storage and/or transport of metals in the plant (i.e., metallothionein, blue copper binding protein and chemocyanin) (Table 2). Upregulation of genes for synthesis of the wheat phytosiderophore, deoxymugenic acid (DMA), which is secreted from wheat roots in greater abundance for plants grown with CuO NPs (McManus 2016), was not observed in the leaf tissue (Table 2).

Activation of genes involved in metal homeostasis was not the only protective pathway upregulated. A battery of genes connected to functions in plant defense also was upregulated (Table 3). The increases in transcript accumulations were more pronounced with growth of the colonized seedlings with CuO NPs than ZnO NPs. Transcriptional changes with *Pc*O6 alone were low (Table 3).

Q-PCR analysis for transcript abundance of selected genes in RNA extracted from leaves generally confirmed the transcriptome findings (Table 4). Gene expression, in leaves of seedlings grown with only CuO NPs allowed analysis of transcriptional changes that occurred from NP-exposure in seedlings lacking *Pc*O6 colonization. Notable were the results for expression from genes encoding a metal-binding chemocyanin, a defense protein (Proteinase Inhibitor Protein, PIP), a lipoxygenase and phosphoethanolamine methytransferase, where synergism resulted in enhanced transcription for plants colonized with *Pc*O6 and exposed to NPs (Table 4). Three genes reported in the microarray analysis to be down regulated also accumulated at a lesser level than in the control plants when assessed by Q-PCR (Table 4).

The microarray data for genes upregulated only by *Pc*O6 colonization illustrated the lack of full annotation for the wheat genome (Supplemental data, array and Tables 2.3.4). For example, 42 out of 76 probes with upregulation of more than 2.8-fold did not

correspond to ascribed functions (Supplemental Table 2). However, genes with predicted functions included those encoding a peroxidase, expansins and metal-transport functions (Supplemental Table 2). Among the most activated genes for the *Pc*O6-colonized seedlings grown with ZnO NPs (Supplemental Table 3) were those encoding changes in genes for LEA proteins and dehydrins, associated with water stress as well as pathogen-resistance functions. A gene for the synthesis of the metal chelator, nicotianamine, was upregulated. Genes with the highest degree of activation by CuO NPs in the *Pc*O6-colonized plant (Supplemental Table 4) similarly encoded functions predicted to be involved in water stress: LEA proteins, dehydrins, proteins designated to be controlled by the water stress regulator, ABA and osmolytes.

Plant root morphology after growth of *Pc*O6 colonized roots with CuO NPs or ZnO NPs and a drought period

Plasticity in root architecture was observed for wheat seedlings colonized by *Pc*O6 when grown with CuO NPs and ZnO NPs. For plants harvested after 7 d growth with normal hydration and 6 d drought, growth with *Pc*O6 but without NPs produced roots that were more robust and had more lateral roots than the seedlings grown as controls (Fig. 4A). *Pc*O6-colonized roots but not shoots were shortened by the presence of ZnO NPs and, to a greater extent, by growth with CuO NPs (Fig. 4A, Supplemental Fig. 2). Lateral roots were abundant in the *Pc*O6-colonized seedlings grown with ZnO NPs (Fig. 4A). Visualization of root hair formation by light microscopy (x 20) showed that the zone of differentiation was not close to the root tips in the plants grown with ZnO NPs, unlike those grown with CuO NPs (Fig. 4B). The *Pc*O6-colonized roots grown with CuO NPs strongly retained sand grains even after rinsing in water because of extensive

root hair formation (Fig. 4A). SEM imaging of root tips (Fig. 4C) confirmed these findings of root hairs close to the root tip only for roots grown with CuO NPs.

Accumulations of metal in the shoot tissue

Because the microarray data showed changes in shoot gene expression related to heavy metal stress we examined the levels of Cu and Zn in dried shoot tissues. There was about a two-fold increase in Cu from growth with CuO NPs and a much larger increase, over ten-fold, in Zn from growth with ZnO NPs (Fig. 5).

Discussion

Tolerance to drought stress was maintained in the plants with roots colonized by *Pc*O6 when grown with NPs at levels that caused changes in root morphology. All seedlings grown with *Pc*O6 had less shoot wilting and higher water contents. This enhanced drought tolerance correlated with systemic increases in transcripts involving proteins regulated by ABA and other predicted protective functions against water stress. Genes encoding protective LEA proteins and dehydrins, water transport and the synthesis of an array of osmolytes (Iordachescu and Imai 2008; Vaseva et al. 2010; Zhou et al. 2012; Kosova et al. 2014; Kurepin et al. 2015; Li et al. 2015; Ding et al. 2016) were upregulated in shoots of the *Pc*O6-colonized plants grown with the NPs. These findings parallel the transcript changes seen in *Arabidopsis* for drought tolerance induced by *Pc*O6 in droughted plants (Cho et al. 2008, 2013). Currently two mechanisms involved with *Pc*O6-induced drought tolerance are known. One is the production by *Pc*O6 of the volatile 2R-3R, butanediol, which triggers the partial closure of stomata (Cho et al. 2008). The second is the formation of biofilms on the plant roots that provide hydration

to the covered plant cells from the gel-like matrix embedding the cells (Timmusk et al. 2015).

Q-PCR analysis of transcript levels for certain genes revealed much higher expression when *Pc*O6-colonized plants were grown with CuO NPs than when noncolonized plants were grown with CuO NPs alone. We speculate that this result is consistent with the priming action of *Pc*O6 that promotes induction of protective genes after a challenge (Kim et al. 2004; Martinez-Medina et al. 2016). The mechanisms accounting for primed induction for systemic effects await resolution but could relate to both *Pc*O6 and the NPs triggering in the root a common stress cue, ROS cell signaling (Mendoza-Soto et al. 2012; Hossain et al. 2015).

The induced transcriptome responses reported here for the leaf tissues differed from the reduction in transcript levels detected with colonization of root tissues for CuO NP-grown plants (Wright et al. 2016). Protective interactions occurring at the root surface could involve production of the *Pc*O6-siderophore, pyoverdine, limiting metal interactions with the plant root, as discussed by Rajkuma et al. (2012). Additionally, the *Pc*O6-biofilms on the plant root could act as a physical shield restricting access of NPs or released metal to the roots, thus, lessening the induction of metal-associated transcripts. Further the use of the acids in the root exudates, citrate and malate, as carbon sources by *Pc*O6 cells could reduce Cu chelates that might be taken up by the plant (McManus 2016; Wright et al. 2016). Indeed, there was a trend for reduced Cu in plants grown with CuO NPs by *Pc*O6-root colonization compared to leaves from NP-grown plants without colonization, agreeing with the findings presented in Wright et al.

(2016). In contrast, *Pc*O6 colonization increased Zn loading from ZnO NPs perhaps due to enhanced NP dissolution.

Induced tolerance to drought was not affected by the changes in wheat root morphology observed from microbial colonization and exposure to NPs. There are likely many interactive players behind the formation of more robust roots with increased lateral root formation observed in the *Pc*O6-colonized plants. Changes in IAA levels due to *Pc*O6 metabolism of root-supplied tryptophan (Adams et al. 2016) or responses to acyl homoserine lactones (AHSLs) produced by the bacterium (Goodman et al. 2016) are two of these possibilities. AHSLs increased lateral root formation in barley (Rankl et al. 2016), a process accompanied by enhanced nitric oxide (NO) accumulation. In *Arabidopsis thaliana,* lateral root formation involves changes in the status of reactive oxygen species (ROS) and specific peroxidase activities (Fernandez - Marcos et al. 2017). Other research implicated IAA flux in lateral root initiation (Talboys et al. 2014; Scheres and Laskowsk 2016; Simonini et al. 2016), perhaps involving modified levels of the auxin efflux inhibitor, cinnamic acid (Steenackers et al. 2017). The colonization of the roots with *Pc*O6 did not prevent the NP-induced changes in root morphology. Increases in lateral roots formation with ZnO NPs confirmed prior observations (Watson et al. 2015; Nair and Chung 2017) but this work showed it occurred without proliferation of root hairs near the root tip. The proliferation of root hairs with Cu exposure has been ascribed to Cu altering IAA flux at the root tip (Yuan et al. 2013). These differential responses to the metals provided as metal oxide NPs indicate that future studies involving defined Zn and Cu levels may generate novel insight on plant signaling in the

root tip that governs root morphology. The linkage between Zn and Cu levels and root morphology also is relevant to formulations of NPs as fertilizers.

Other wheat genes that responded to NPs for the colonized plant included those involved in responses to heavy metals and ROS. These findings align with accumulations of Cu or Zn in shoots of colonized plants grown with CuO or ZnO NPs. Increased expression of genes involved in nicotianamine synthesis agreed with the role of this compound as a chaperone for Zn and Cu transportation. Nicotianamine is present in the phloem of wheat (Palmer et al. 2014) and Cu- or Zn-nicotianamine complexes have been found in the phloem of rice (Yoneyama et al. 2015; Zheng et al. 2012). The lack of activation of genes for the synthesis of the phytosiderophore, DMA, in the leaf tissue might be expected of genes that were only expressed in root cells. We have detected DMA in rhizosphere solutions of wheat seedlings grown with CuO NPs and a role for DMA chelation of Cu outside of the root at high Cu levels has been suggested (McManus 2016). Transport of both Cu- and Zn-DMA derivatives in rice phloem has been documented (Yoneyama et al. 2015), suggesting that an intact metal-DMA complexes can be transported from the root to other plant tissues. Transport as nicotianamine or DMA complexes could be involved in the systemic metal activation of gene expression in the shoots. Stronger induction of genes encoding metal-binding proteins (metallothionein family proteins and the chemocyanins) by seedling growth with CuO NPs compared to ZnO NPs may relate to the higher phytotoxicity of Cu than Zn. Other genes were upregulated more by wheat growth with ZnO NPs than with CuO NPs indicating that the plant responded differently to the two metal oxides. It is interesting that Zn is transported into the vacuoles of plant cells (Olsen and Palmgren 2014) so that

intracellular trafficking of Zn complexes is required. The exact roles for these metal binding proteins in plants are not resolved but metal storage and chelation for transport as a protected complex is proposed (Cobbett and Goldsbrough 2002).

The changes in gene expression relating to responses to heavy metal stress correlated with enhanced Cu levels in the wheat shoot when plants were grown with the NPs. The increase of about 2-fold from growth with CuO NPs was much less than the change observed with exposure to ZnO NPs. Both CuO NPs and Cu ions should be transported within the plant (Wang et al. 2012; Dimkpa et al. 2013). Thus, the accumulation of Cu in the leaves could have been limited by entrapment on the root cell walls as discussed by Peng et al. (2015). It will be interesting to determine whether the speciation of Cu within shoot tissues detected by XANES analysis varied between colonized and noncolonized roots. Additionally, the high Zn levels were consistent with greater dissolution potential of the ZnO NPs than the CuO NPs (Wang et al. 2013). Dissolution could perhaps have been aided by *Pc*O6 metabolites. The observation of high uptake of Zn into wheat contrasted with the findings in *A. thaliana* that Zn from ZnO NPs was not transported into shoots (Nair and Chung 2017). Thus, there are differences between plants in their responses to the same NPs.

These findings illustrate that CuO and ZnO NPs will interact with root-colonizing microbes and the interplay will alter plant growth and function under stress. The work illustrates the roles of Cu and Zn availability in governing root morphology and function. Such interactions will be important in determining how these NPs can be included in agricultural formulations.

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Figure legends

Figure 1. Effect of 6 d-drought on leaf wilting for wheat seedlings first grown for 7 d under normal hydration. Images representative of results from three different studies each with five replicates per treatment and boxes planted with 25 seeds are shown. The treatments were control, with *Pc*O6 root colonization, with *Pc*O6 root colonization and exposure to NPs (300 mg Cu/kg sand from CuO NPs or 500 mg Zn/kg sand from ZnO NPs). The images are typical of three separate studies.

Figure 2. Effect of growth of wheat seedlings, with and without colonization by *Pc*O6 and with and without NPs (CuO, 300 mg Cu/kg and ZnO 500 mg Zn/kg), on leaf water content. Plants were grown with normal hydration for 7 d and then water was withheld for 6 d. The data shown are fresh weight of leaves/seedling and the loss of water/seedling upon drying the leaves. The data are means for two studies each with five replicates/treatment with 25 seeds/box.

Figure 3. Venn diagrams summarizing the transcriptome data set showing the numbers of genes that were unique to a treatment as well as the numbers that overlapped from RNA extracted from 7 d wheat seedling leaves after growth with *Pc*O6-colonized roots and exposure to CuO NPs or ZnO NPs (300 mg Cu/kg sand from CuO NPs or 500 mg Zn/kg sand from ZnO NPs).

Figure 4. A. Plant root morphology after growth (7 d with hydration and 6 d drought) with no treatments (control), colonization by *Pc*O6, or colonization by *Pc*O6 with

exposure to CuO NPs or ZnO NPs. The NPs were added to the sand growth mix at 300 mg Cu/kg or 500 mg Zn/kg dry sand from the NPs. Images of two roots for each treatment, typical of 75 roots grown under the same conditions are shown. **B.** Estimations of the positioning of root hairs on root tips of 7 d-old *Pc*O6–colonized wheat seedlings raised in the presence and absence of NPs (300 mg Cu/kg sand from CuO NPs or 500 mg Zn/kg sand from ZnO NPs). The data presented as % values with standard deviations are from a minimum of 30 roots from three studies for each treatment. The distance between the root tip to the point where root hairs were first observed was graded into three regions. The evaluation was performed at x20 magnification with the fresh root tips being immersed in water. **C**. SEM imaging of wheat seedling roots to compare root tip morphology when grown with *Pc*O6 colonization for 7 d in the presence or absence of NPs (300 mg Cu/kg sand from CuO NPs or 500 mg Zn/kg sand from ZnO NPs). Data shown are typical of 3 root tips for each treatment from two different studies.

Figure 5. Metal accumulation into shoot tissue. Loads were determined in shoots, without coleoptiles, from seedlings grown for 7 d with hydration followed by 6 d drought. Seedlings were grown with or without PcO6 colonization and with or without CuO MPs or ZnO NPs (300 mg Cu/kg sand from CuO NPs or 500 mg Zn/kg sand from ZnO NPs). The * denotes difference between control and NP treatment (P=0.01). The** denotes difference between the loads with and without *Pc*O6 colonization for that treatment (P=0.05). Data are mean and standard deviations for two studies each with five replicates of each treatment and 25 seeds/box.

Figure 1.

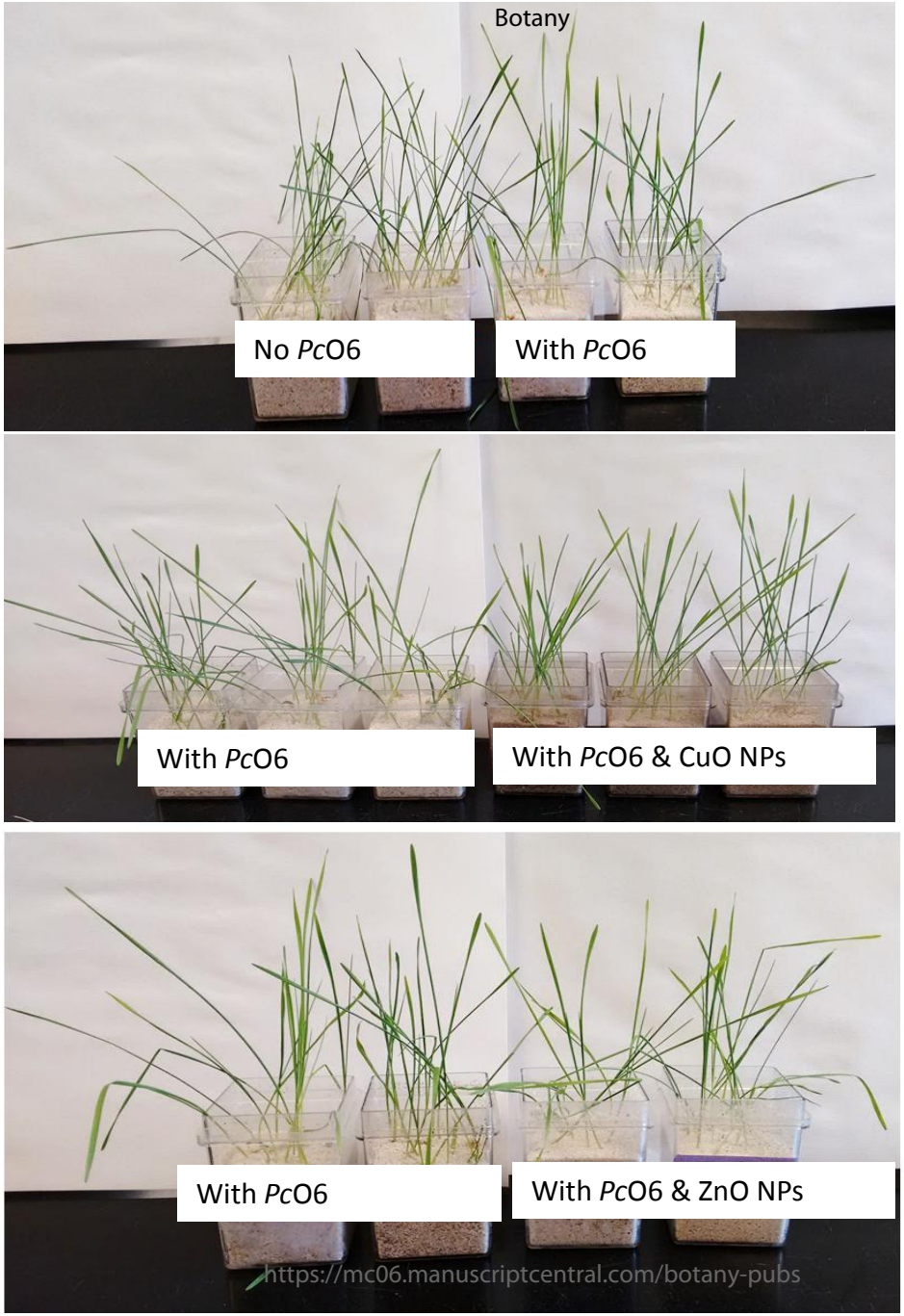

No *Pc*O6
With *Pc*O6
With *Pc*O6
With *Pc*O6 & CuO NPs
With *Pc*O6
With *Pc*O6 & ZnO NPs

Figure 2.

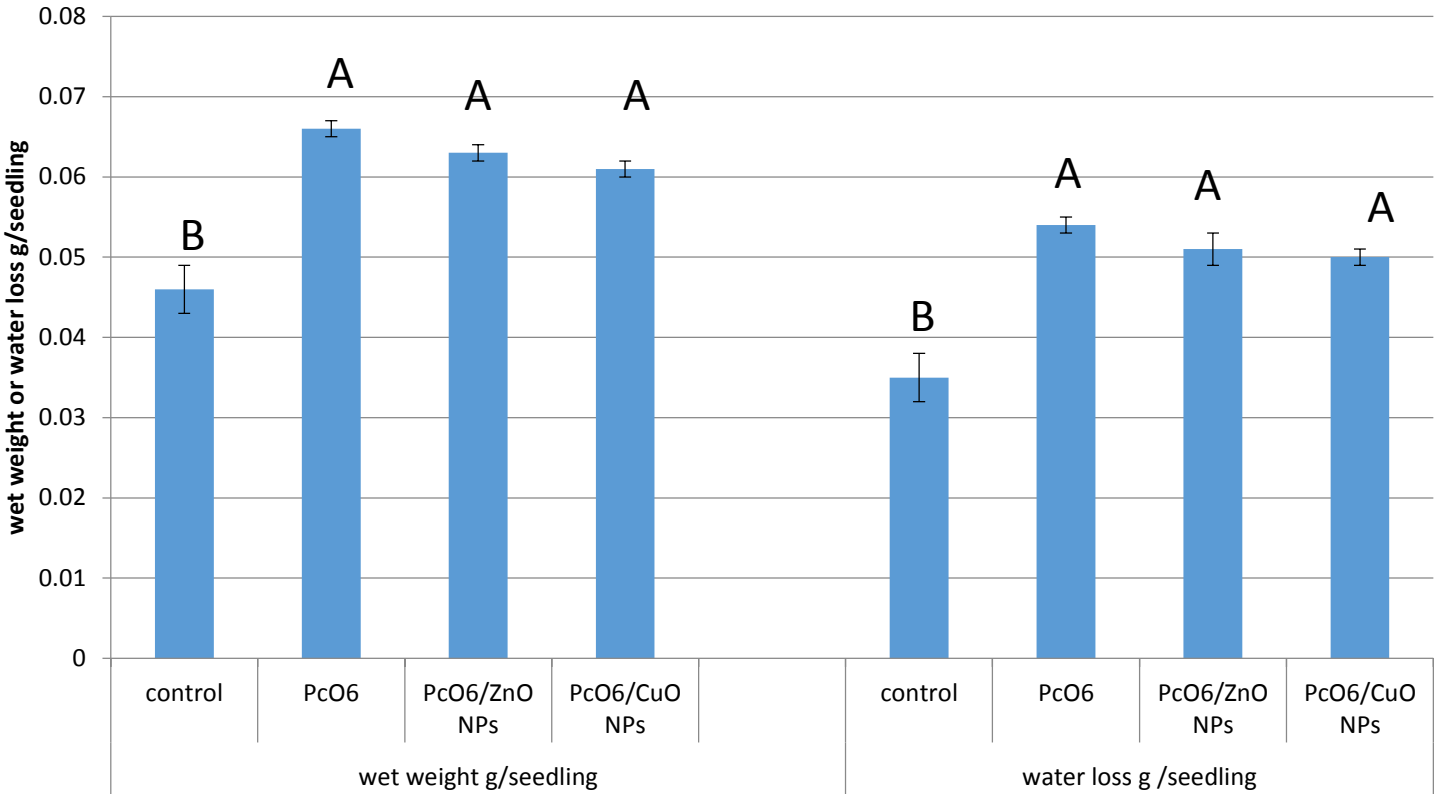
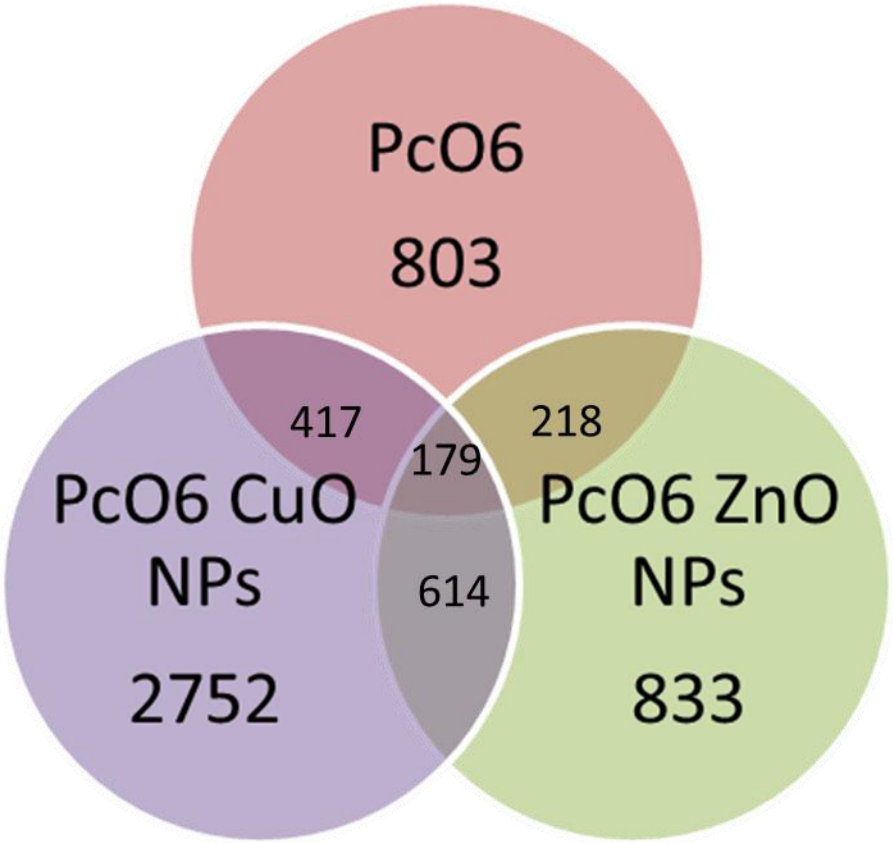
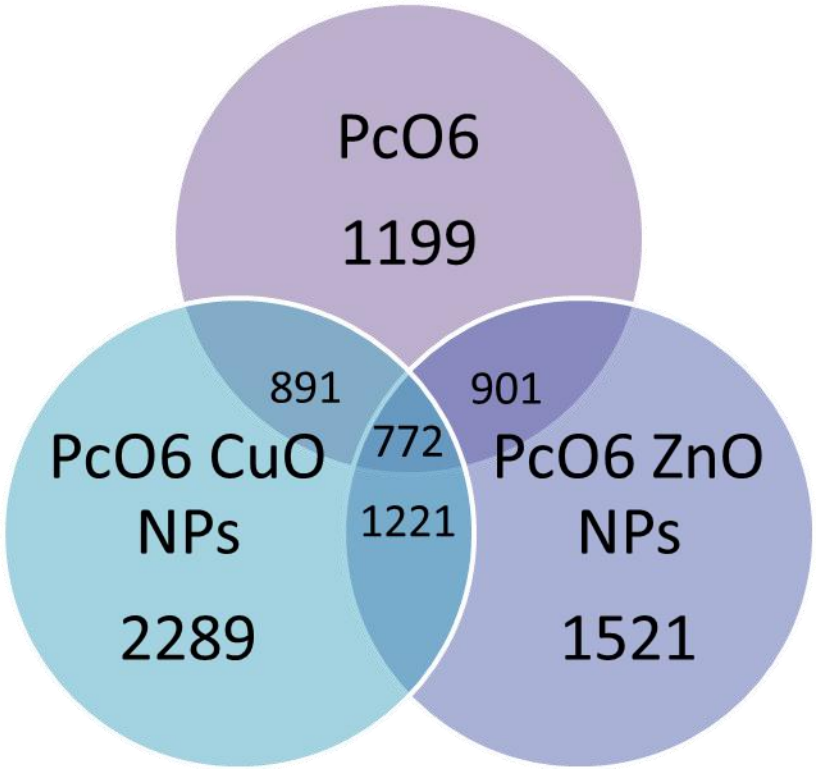

wet weight or water loss g/seedling
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
B
A
A
A
B
A
A
A
control
PcO6
PcO6/ZnO NPs
PcO6/CuO NPs
control
PcO6
PcO6/ZnO NPs
PcO6/CuO NPs
wet weight g/seedling
water loss g /seedling

Figure 3.

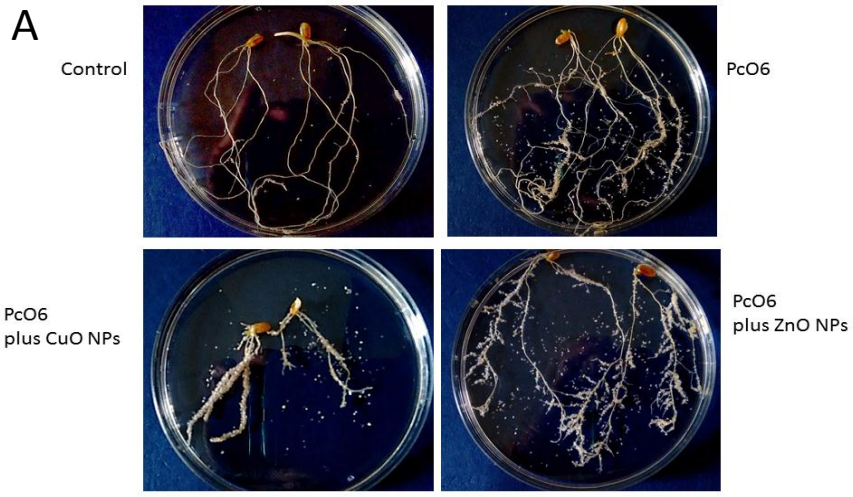


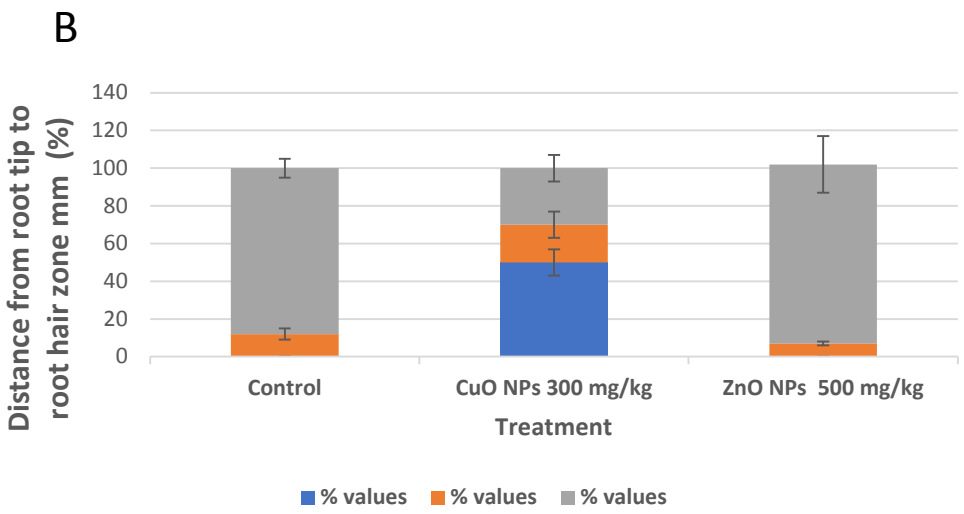
Up-regulated genes



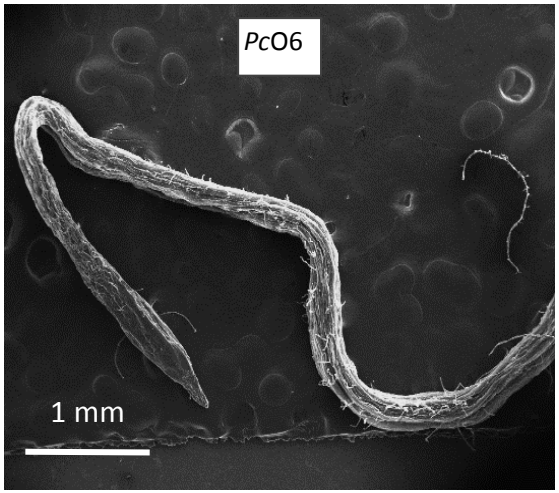
Down-regulated genes

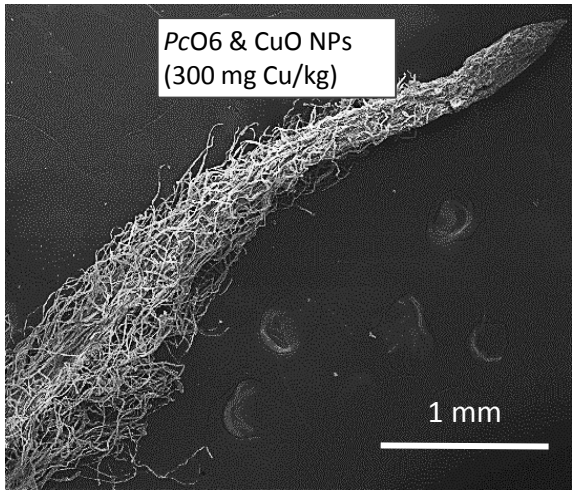
Fig.4


A
Control
PcO6
PcO6
plus CuO NPs
PcO6
plus ZnO NPs


B
Distance from root tip to root hair zone mm (%)
140
120
100
80
60
40
20
0
Control
CuO NPs 300 mg/kg
ZnO NPs 500 mg/kg
Treatment
% values
% values
% values

C


PcO6
1 mm


PcO6 & CuO NPs
(300 mg Cu/kg)
1 mm

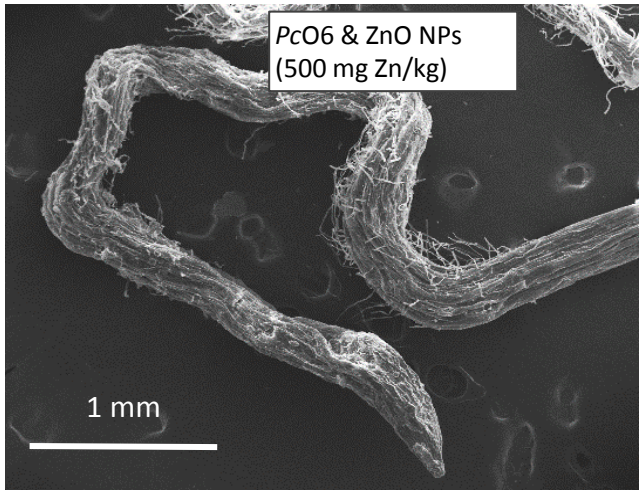

PcO6 & ZnO NPs
(500 mg Zn/kg)
1 mm

Figure 5.

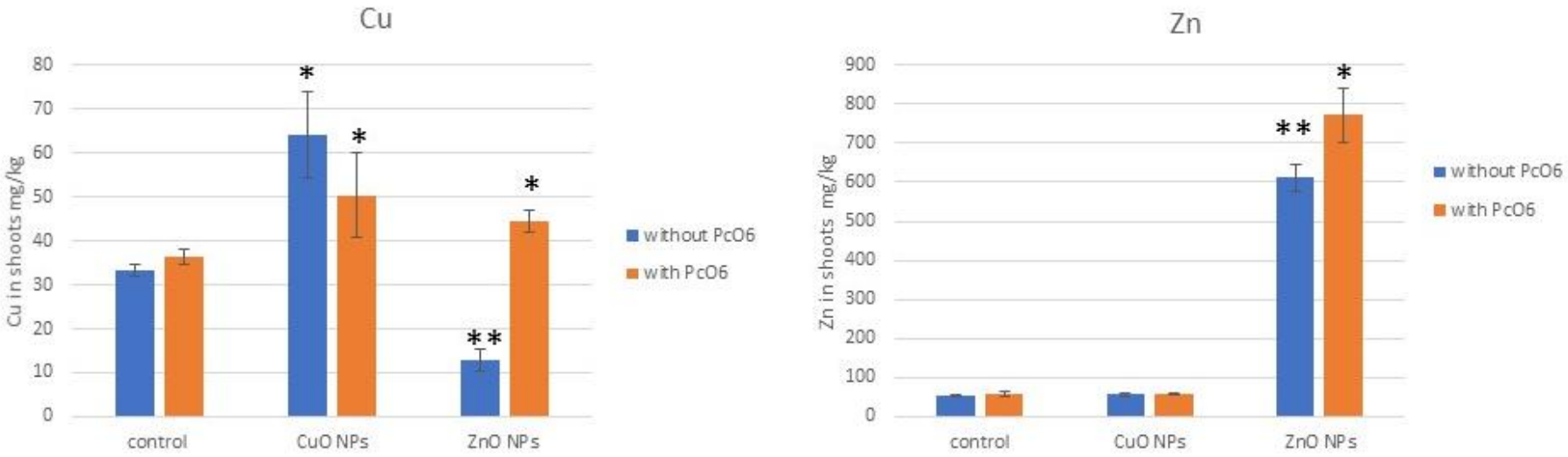

Cu
Cu in shoots mg/kg
80
70
60
50
40
30
20
10
0
control
CuO NPs
ZnO NPs
without PcO6
with PcO6
Zn
Zn in shoots mg/kg
900
800
700
600
500
400
300
200
100
0
control
CuO NPs
ZnO NPs
without PcO6
with PcO6

Table 1. Up-regulation of genes associated with water stress in leaf tissue by growth of *Pc*O6-colonized wheat seedlings with NPs

Probe name	Potential predicted function in wheat	CuO NPs & *Pc*O6	ZnO NPs & *Pc*O6	*Pc*O6
Proteins predicted to be related to water stress				
A_99_P289696	LEA protein	81.4	15.1	1.12
A_99_P036114	LEA protein	43.4	6.1	2.3
A_99_P167849	Cold acclimation protein WCOR80 ABA	15.5	8.6	-1.3
A_99_P384227	Dehydration responsive element protein	11.3	4.6	1.1
A_99_P580477	Dehydrin WZY1-1	11.4	5.6	1.1
A_99_P218621	ABA inducible protein	11.3	2.2	1.0
A_99_P074020	LEA2 protein	11.1	2.2	1.0
A_99_P330946	Cytokinin dehydrogenase -	10.8	6.3	1.3
A-99_P218691	LEA 3 protein	6.1	1.05	0.7
A_99_P029139	Salt stress protein	6.3	2.3	1.1
A_99_P315801	Water stress related protein	6.1	2.7	-1.3
A_99_P218691	LEA3 protein	6.1	1.8	1.0
A_99_P186492	Wrab18 ABA inducible protein	5.9	2.5	-1.12
A_99_P485987	LEA1 protein	5.1	1.3	-1.1
A_99_P240051	Dehydrin	4.2	3.3	0.6
A_99_P400397	ABA responsive element factor binding protein	2.9	1.9	0.4
Predicted function in osmolyte production				
Fructan metabolism				
A_99_P133905	Sucrose:fructan 6-fructosyltransferase -	13.3	2.3	1.4
A_99_P560262	Sucrose:fructan 6-	11. 0	2.0	1.3

	fructosyltransferase			
A_99_P299401	Fructan 6-exohydrolase	6.2	-1.0	1.3
A_99_P503042	Fructan:fructan 1-fructosyltransferase	5.7	1.5	1.1
A_99_P376782	Trehalose phosphate synthase	2.03	0.51	1.60
Putrescein formation				
A_99_P413662	Ornithine/acetylornithine aminotransferase	4.83	2.75	1.35
A_99_P174104	Ornithine/acetylornithine aminotransferase	3.95	2.80	1.19
A_99_P301751	Ornithine decarboxylase antizyme inhibitor	7.90	1.30	1.10
Predicted function in glycine betaine formation				
A_99_P266341	Betaine aldehyde dehydrogenase	2.12	1.44	1.10
A_99_P266336	Betaine aldehyde dehydrogenase	2.43	1.22	1.55
Predicted function in water movement				
A_99_P235211	Aquaporin	3.15	1.50	1.70
A_99_P430387	Aquaporin	2.61	1.82	1.07
A_99_P235211	Aquaporin	3.15	1.70	1.50
Predicted function in cell wall modifications				
A_99_P080280	Expansin	5.50	2.05	-1.02
A_99_P000361	Expansin	12.50	1.48	6.64
A_99_P238191	Expansin	3.45	2.74	2.35

Table 2. Genes with predicted function in metal homeostasis that are upregulated in leaf tissue by growth of *Pc*O6-colonized wheat seedlings with NPs

Probe number	Fold change CuO NPs & *Pc*O6	Fold change ZnO NPs & *Pc*O6	*Pc*O6	Gene function
A_99_P355421	6.72	4.98	1.57	Nicotianamine synthase
A_99_P200801	2.77	2.47	2.11	Nicotianamine synthase
A_99_P437347	2.35	-1.27	2.99	Nicotianamine synthase
A_99_P200621	2.50	1.74	2.83	Nicotianamine synthase
A_99_P200591	2.00	1.36	-1.28	Nicotianamine Synthase
A_99_P463432	3.23	1.72	1.10	Metallothionein TC412708
A_99_P225551	3.73	1.07	1.61	Metallothionein TC438375
A_99_P440802	2.59	1.75	1.10	Metallothionein TC397006
A_99_P005136	2.43	1.07	1.92	Metallothionein TC409201
A_99_P302311	2.30	1.79	1.78	Metallothionein TC418368
A_99_P302311	2.10	1.77	2.49	Metallothionein TC402210
A_99_P156377	2.20	2.15	-1.12	Metallothionein

				TC397006
A_99_P467187	2.07	1.63	1.33	Metallothionein TC414908
A_99_P200731	10.73	3.46	1.43	Blue Cu chemocyanin like
A_99_P197481	9.49	2.77	1.17	Basic blue copper protein
A_99_P291776	1.21	1.16	-1.02	DMA synthase
A_99_P012349	1.11	1.12	-1.13	DMA synthase

Table 3. Up-regulation of genes associated with plant defense in leaf tissue by growth of *Pc*O6-colonized wheat seedlings with NPs

Plant defense genes probe name	Putative function	CuO NPs & *Pc*O6	ZnO NPs & *Pc*O6	*Pc*O6
A_99_P254221	defensin	35.4	4.3	2.8
A_99_P167814	glucan endo-1,3-beta-D-glucosidase	21.5	1.5	2.9
A_99_P453277	thaumatin like protein	21.3	-1.0	1.2
A_99_P466672	PR1a	21.2	2.4	2.3
A_99_P268711	xylanase inhibitor-like	15.8	4.8	3.1
A_99_P210941	endo-beta-1,3-glucanase	14.0	2.0	2.2
A_99_P011059	stress inducible protein	12.9	2.0	1.4
A_99_P565987	wound signal protein	11.6	7.9	1.1
A_99_P424247	chitinase	10.1	-1.0	1.7
A_99_P234306	wrsi5-1 protein	10.1	2.6	-1.2
A_99_P105360	flavanone 3-dioxygenase	10.1	1.4	-1.1
A_99_P265201	disease resistance protein	9.7	6.3	1.6
A_99_P210426	glucan endo-1,3-beta-D-glucosidase	7.9	1.6	1.7
A_99_P546727	xylanase inhibitor	6.2	3.0	3.3
A_99_P475207	protease inhibitor	5.8	2.4	1.7
A_99_P432267	defensin	5.5	1.7	1.6
A_99_P489822	xylanase inhibitor 602OS	5.1	2.8	3.2
A_99_P198256	endo-beta-1,3-glucanase	5.1	1.3	1.1

Table 4. Q-PCR analyses to determine expression in leaf tissues of selected genes due to challenges of the seedling roots.

	Gene chip: fold changes			**Q RT PCR: fold changes**				
Gene	***Pc*O6**	**CuO NPs & *Pc*O6**	**ZnO NPs & *Pc*O6**	**Control**	***Pc*O6**	**CuO NPs**	**CuO NPs & *Pc*O6**	**ZnO NPs & *Pc*O6**
Genes with metal-associated function								
Metallothionein (A_99_P225551) AY736124	1.07	3.73	1.61	1	1.5	2.8	2.2	1.1
Chemocyanin (A_99_P200731) GenBank: AK330966, JV908908	1.43	10.73	3.46	1	3.1	2.4	41	6.4
Blue copper binding protein (A_99_P226086)	-2.13	-4.54	-3.62	1	0.33	0.62	0.47	0.52

FJ459810								
Genes associated with defense functions								
PIP (A_99_P234306) GenBank: AY549888	-1.25	10.2	2.64	1	0.75	1.75	44	1.1
β-glucanase (A_99_P167814) GenBank: DQ090946		2.95	1.56	1	0.9	2.78	1.84	1.53
Lipoxygenase (A_99_P234306) GenBank: AK332064.1	-1.85	4.5	2.0	1	0.60	4.5	5.85	1.4
Genes associated with general stress responses								
Phosphoethanolamine methyltransferase (A_99_P223461) GenBank: AK332332.1	1.36	8.31	3.67	1	1.44	1.25	7.89	0.86

ABCG3 (A_99_P295716) GenBank: JN800000	-1.98	-6.23	-2.07	1	0.81	0.25	0.10	1.18
GS transferase (A_99_P423722) GenBank: JX051004	-4.22	-31.1	-8.23	1	1.88	0.17	0.01	1.0