Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supporting Information

Copper sulfide nanoparticles suppress *Gibberella fujikuroi* infection in *Oryza sativa* seeds by multiple mechanisms: Contact-mortality, nutritional modulation and phytohormone regulation

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Supplemental experiment

Experiment S1. CuS NP synthesis and characterization

The procedures for CuS NP synthesis were from Li et al., with some modifications.[1] Briefly, CuO NP (< 50 nm, Sigma-Aldrich Chemistry, MO, USA) stock solutions were prepared in a 250 mL glass beaker containing 500 mg of CuO NPs (6.25 mM Cu) and 200 mL of nanopure water. Nanopure water produced by ultrapure water system (Barnstead, IA, USA) was used for material synthesis. The NP-suspensions were sonicated at 12 W for 20 mins using a sonic dismembrator (Fisher scientific, NH, USA). Afterwards, 6.25 and 25 mM Na_2S were added into the CuO NP stock solutions to make the molar ratio of Cu/S at 1 and 4, respectively. CuS NPs with the molar ratio of Cu/S at 1 and 4 were defined as CuS (1:1) NPs and CuS (1:4) NPs, respectively. The mixtures were shaken horizontally at 100 rpm on an orbital shaker at ambient temperature (20 ± 2 °C) for 48 hours. The pH was kept at 10.0 ± 0.2 in order to form the target compounds. The reaction solutions were centrifuged at 9000 rpm for 30 mins and repeatedly washed with nanopure water for 6 times. The synthesized compounds were freeze-dried prior to use.

The morphology and size of Cu-based NPs were observed using transmission electron microscopy (TEM) (JEOL 200CX, Jeol Inc., Tokyo, Japan) operated at 200 kV. Briefly, 500 mg/L NP-suspensions were diluted 20 times in nanopure water and then mounted onto nickel grids to dry. NP crystal structures were measured by X–ray diffraction (XRD) and data were collected with continuous scanning 2θ from 20° to 80° at 0.0263° per step. The initial hydrodynamic diameter (D_h) and zeta potential of 25 mg/L of each Cu-based NPs were characterized at 25 °C by a 90 Plus particle size analyzer (Brookhaven, NY, USA).

TEM images show that the size of CuS (1:1) and CuS (1:4) NPs were significantly smaller than CuO NPs; CuS (1:1 and 1:4) NPs possessed many particles in the 5-10 nm range (**Figure S1A, B and C**). The average hydrodynamic diameter of CuO, CuS (1:1), and CuS (1:4) were 309±0.69, 249±0.5 and 252±0.18 nm, respectively (**Figure S1D, E, and F**), all of which were significantly smaller than Kocide 3000 (608±0.5 nm, **Figure S1G**). The sulfidation of CuO NPs was highly dependent on the starting molar ratio of Cu to sulfide. When the Cu:S ratio was at 1:1, which is the theoretical amount of sulfide for stoichiometric conversion, the sulfidation process was incomplete as determined by XRD (**Figure S1H**); characteristic peaks of crystalline CuO were still evident. At the Cu/S ratio of 1:4, no crystalline CuO was evident, indicating that the conversion was essentially complete.

Experiment S2. Conidial suspension collection

A representative isolate of *G. fujikuroi*, FGSC #8381, was stored in potato dextrose agar (PDA) (Fisher Scientific, NH, USA) at –20 °C. A small plug was transferred onto V8 juice agar medium (20% (*v/v*) V8 vegetable juice, 3 g $CaCO_3$, and 2% (*w/v*) agar in one liter) and was incubated at 25 °C for 10 days to allow mycelia to cover the medium surface and sporulate.[2] Conidia were collected from 10-day old sporulating mycelia by adding 20 mL of sterilized nanopure water into petri dishes, and the mycelia were gently rubbed off the medium with a sterile glass rod. These conidial suspensions were filtered through a sterile muslin cloth to remove the fraction of mycelia.[3] The conidial suspensions were diluted with sterile nanopore water and the approximate number of conidia was determined using a hemocytometer. The final cell concentration was at 10^6 conidia per milliliter. These pure conidial suspensions were stored at 4 °C until further use.

Experiment S3. Dose optimizing for *G. fujikuroi* control *in vitro*

Prior to investigating the antifungal activity of the in-house CuS NPs, commercial CuO NPs were used to determine the optimum dose for *G. fujikuroi* control. Upon exposure to CuO NPs at 10-250 mg/L over 7 days, colony-forming units (CFU) and dehydrogenase activity of *G. fujikuroi* were measured. Upon CuO NP exposure, a dose-dependent loss in *G. fujikuroi* cell viability was evident (**Figure S2A-C**). In the untreated control, *G. fujikuroi* CFU increased slowly in the first 24 hours, followed by a rapid increase between 48 and 72 hours (**Figure S2A**). Upon treatment with 10 mg/L CuO NPs, *G. fujikuroi* growth was greatly slowed, with essentially no growth after 72 hours. At 50 mg/L, the significant decrease in the *G. fujikuroi* CFU was evident after 72 hours, although fungal growth was still evident during the first 72 hours. At 150 and 250 mg/L, CuO NPs completely inhibited the fungal growth. Thus, 50 mg/L was chosen as the dose for subsequent experiments. Additionally, the pattern of dehydrogenase activity of *G. fujikuroi* at 168 hours across all treatments were consistent with the fungal growth data (**Figure S2C**).

Experiment S4. Dehydrogenase activity

At Day 7, 2 mL of *G. fujikuroi* culture from each of the treatments were transferred into an Eppendorf tube and centrifuged at 4,000 rpm at 4 °C for 15 min. The pellet was re-suspended in 2 mL of deionized H_2O, and 2 mL of 0.2% (*w/v*) triphenyl tetrazolium chloride (TTC) were added to the suspension. The mixture was inoculated at 37 °C overnight. Any red formazan that formed was extracted by 4 mL acetone and was quantified on a UV–Vis spectrophotometer (Agilent, CA, USA) at 484 nm.[4]

Experiment S5. Dissolution

The dissolution rate of 50 mg/L Cu-based particles was evaluated in nanopore water and PDB with conidia. All samples were shaken at 110 rpm at 25 °C in an incubator shaker (Marshall Scientific, Cambridge MA) and 0.5 mL of aliquots were sampled at specific intervals (12, 24, 48, 72, and 168 h). NPs were removed from suspension by centrifugation at 13,000 rpm, and the supernatant was filtered by Amicon® Ultra-15 centrifugal filter devices (Molecular weight cutoff: 3k Da). The Cu concentration was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, PerkinElmer 4300 Dual View, Shelton CT). There were four replicates in each treatment. The recovery of $CuSO_4$ solution in Amicon® Ultra-15 centrifugal filter devices (Molecular weight cutoff: 3k Da) was 99.60% ± 2.13%. The Cu recovery in rice was 101.82 ± 6.31%. The Cu standard curve was made by different concentrations of Cu solution (0-50 mg/L), and the standard curve was a linear relationship with r^2 at 0.999.

Experiment S6. Rice seed infection by *G. fujikuroi*

Rice seeds (*Oryza sativa* L.) were surface sterilized with 30% (*v/v*) H_2O_2 for 10 min and washed thoroughly with sterile nanopure water. The seed infection procedures were followed as described in Jo et al. with minor modification.[2] Briefly, 500 seeds were soaked in 150 mL of conidial suspension (10^6 conidia per milliliter) and shaken at 120 rpm for 24 hours at ambient temperature (25 °C) to enable conidia attachment to the seed surfaces. An additional 500 seeds were soaked in 150 mL sterile nanopure water under the same conditions to serve as the non-infected control. All seeds were then air-dried on petri dishes for future use.

Experiment S7. Selection of NPs doses and greenhouse conditions

NP doses used in this experiment were optimized by measuring germination and seedling growth of uninfected rice upon exposure to different concentrations of CuO NP suspensions in vermiculite under greenhouse conditions. Briefly, for seed treatment, 20 uninfected seeds were soaked into different concentrations of Cu-based NP suspensions (equivalent to 3, 15, 45 and 75 μg Cu per seed) or sterile nanopure water and shaken at 110 rpm for 15 min, 1 h, 4 h, 8 h and 24 h at room temperature. The seed germination rate suggests that the optimized 15 μg Cu/seed and 24 h should be used in the following experiments (**Figure S3**) as no toxicity to rice seeds was observed under such conditions. For foliar application, the two-week old healthy seedlings were foliar-treated with 50 mg/L NP-suspension or sterile nanopure water as control. The NP-foliar application was applied twice within a 7-day interval and the actual volume transferred onto the seedling of each replicate was 0.5 mL (equivalent to 50 μg Cu/replicate/week). No phytotoxicity was found in five-week old healthy plants that were treated with NPs at applied concentrations (15 μg Cu/seed or 50 μg Cu/replicate/week) in both seed and foliar applications. The temperature in the greenhouse was 25−30/20−25 °C (day/ night) with relative humidity 60%−70%, and a light intensity of 16 500 lx. All plants were fertilized with the following nutrient solution (mmol·L^{-1} in one liter) at 25% strength: K_2SO_4, 0.75; $Ca(NO_3)_2$, 2.0; KCl, 0.1; KH_2PO_4, 0.25; $MgSO_4 \cdot 7H_2O$, 0.65; H_3BO_3, 1.0×10^{-3}; $MnSO_4 \cdot H_2O$, 1.0×10^{-3}; $ZnSO_4 \cdot 7H_2O$, 1.0×10^{-3}; $CuSO_4 \cdot 5H_2O$, 1×10^{-4}; $(NH_4)_6Mo_7O_{24}$,5×10^{-6}; Fe-EDTA, 0.1.[5] One liter of the nutrient solution was supplied every 3 days to supply sufficient nutrition for the plants.

Experiment S8. Evaluation of disease incidence

As symptoms of fungal infection developed, plants were rated for severity on a scale of 0 to 4, where 0 = asymptomatic, 1 = slightly stunted or long internodes, 2 = stunted or long internodes, 3 = dead, 4 = no germination. The disease incidence was calculated by the following equation:

$$\text{Disease incidence} = (\sum S_i \times N_i)/ (4 \times N_{total}) \quad (1)$$

where S_i = the disease rating scale range from 0 to 4; N_i = the number of the plants at disease rate of i; N_{total} = the number of plants in total.[6] Higher values of disease incidence represent greater disease progression and vegetative damage. At harvest, the fresh shoot and root mass were separately determined across all treatments. All tissues were dried in an oven and acid-digested for nutrient analysis.

Experiment S9. Total chlorophyll content determination

Briefly, 50 mg of fresh leaves were cut into pieces and soaked into 10 mL of 95% (*v/v*) ethanol to extract the chlorophyll. All samples were incubated in the dark for 3 d, and the absorbance of supernatant was measured at 664.2 and 648.6 nm by a UV–Vis spectrometer (Agilent, CA, USA). Total chlorophyll was calculated by Chla = 13.36A664.2 − 5.19A648.6, Chlb = 27.43A648.6 − 8.12A664.2, and total chlorophyll = Chla + Chlb.[7]

Experiment S10. Folin-Ciocalteu assay

The total phenolics content was determined by the Folin-Ciocalteu assay.[8] Briefly, 0.4 g ground rice leaf sample was mixed with 10 ml of 80% methanol, and then this mixture was shaken at 110 rpm at room temperature for 12 h to ensure full extraction. After centrifugation at 4000 rpm for 20 mins, the supernatant was collected to determine the total phenolics. In brief, 50

μL of extraction were added into a solution with 450 μL nanopure water and 250 μL of 2 mole/L Folin−Ciocalteu reagent. The mixture was added into 1.25 mL 20 g/L Na_2CO_3, incubated at 25 °C for 20 min, and then centrifuged at 4000 rpm for 10 min. The absorbance of supernatant absorbance was measured at 735 nm using a UV–Vis spectrometer (Agilent, CA, USA). The standard curve was prepared using gallic acid (GA) with a regression $R^2 = 0.998$.

Experiment S11. Quality assurance/quality control (QA/QC) information for phytohormone and phytoalexin measurement

To determine the phytohormone, a series of standards (salicylic acid, jasmonic acid, abscisic acid and sakuranetin; Sigma-Aldrich) at 1, 5, 10, 15, 20 and 25 mg/L were prepared in CH_3OH/H_2O (8:2; *v/v*) as calibration curves for each analyte and the standard curves were a linear relationship with r^2 all above 0.998. In addition, to check the status of the instrument, a standard at 10 mg/L was detected every 20 samples.

Supplemental figures

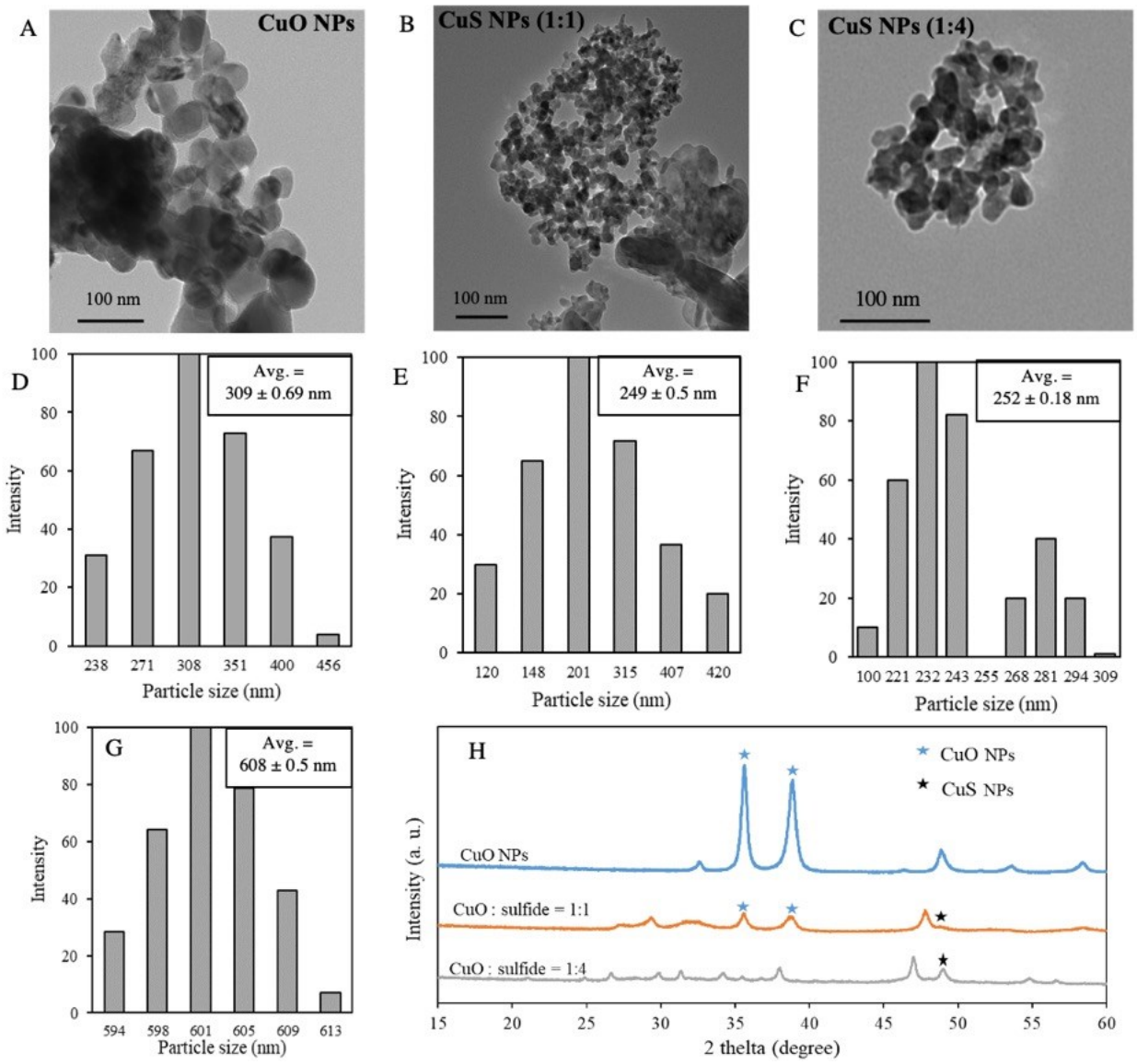

Figure S1. Characterization of the Cu-based NPs. TEM images of Cu-based NPs including (A) CuO NPs, (B) CuS NPs (1:1) and (C) CuS NPs (1:4). Size distribution of Cu-based NPs including (D) CuO NPs, (E) CuS NPs (1:1), (F) CuS NPs (1:4), and (G) Kocide 3000. (H) XRD patterns of Cu-based NPs.

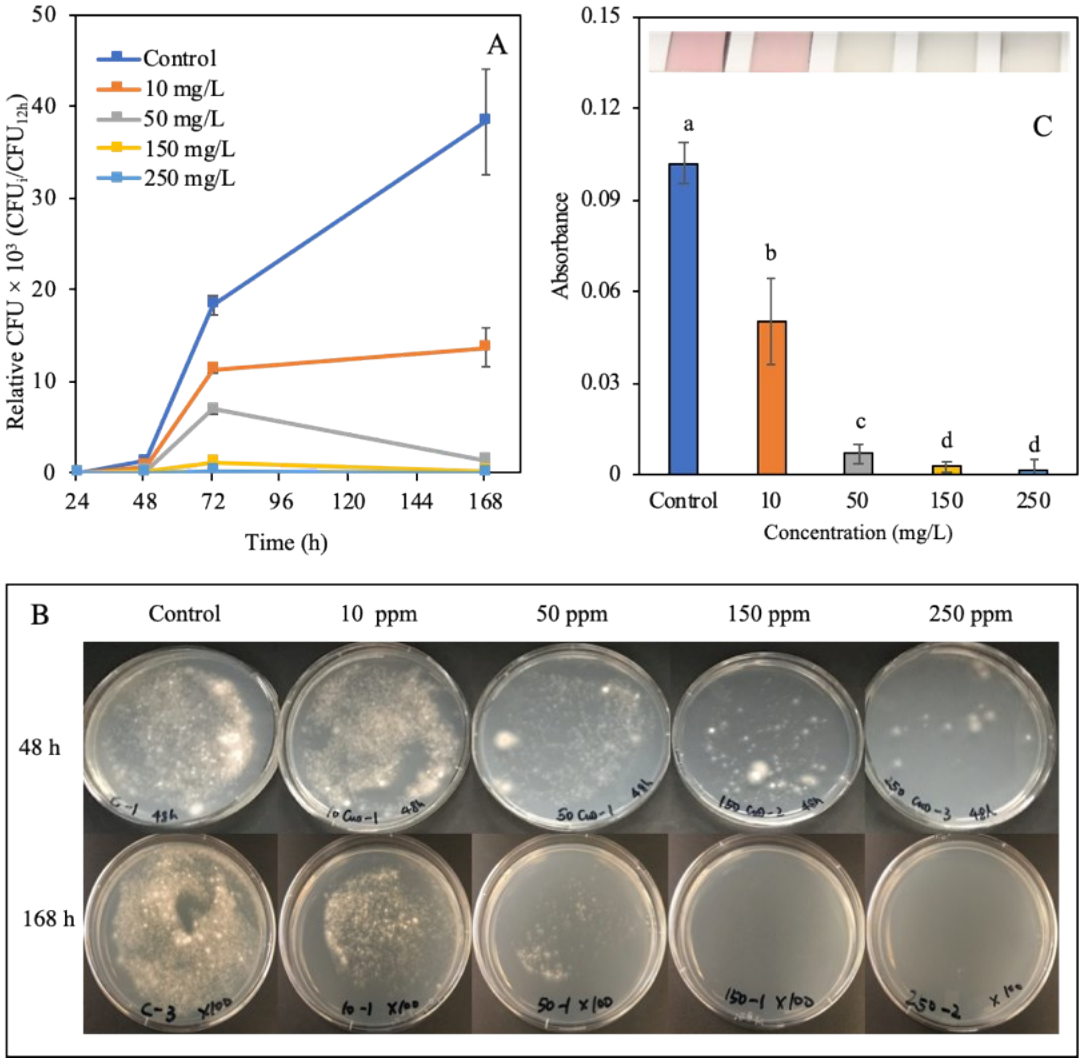

Figure S2. Growth inhibition of *G. fujikuroi* in PDB medium amended with CuO NPs at 10-250 mg/L. (A) Relative CFU counts on petri dishes over 168 hours (a CFU at designed time point over the CFU at 12 hours), (B) The growth of *G. fujikuroi* on petri dishes after treating with CuO NPs in PBD medium for 48 and 168 hours, and (C) Dehydrogenase activity of *G. fujikuroi* after treating with CuO NPs in PBD medium for 168 hours. The means are averaged from four replicates. Error bars correspond to standard error of mean. All values marked with different letters are significantly different at $p < 0.05$.

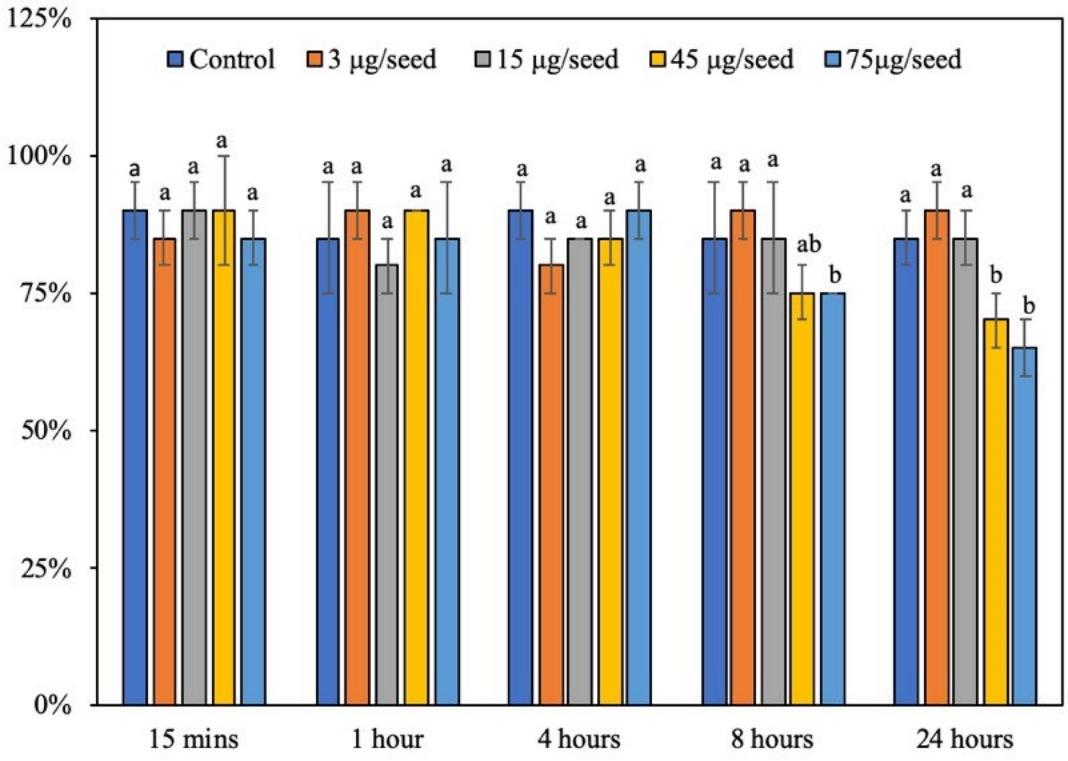

Figure S3. Seed germination rate after seed-exposure to Cu-based NPs for 15 mins-24 hours. The means are averaged from six replicates. Error bars correspond to standard error of mean. All values marked with different letters are significantly different at $p < 0.05$.

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