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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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Environmental significance

Copper (Cu)-based pesticides have been widely used as a broad-spectrum management strategy in sustainable agriculture. However, many years of continual uses have resulted in Cu contamination of soil and surface water. Development on improving the antimicrobial efficiency of Cu-based pesticides while simultaneously minimizing their environmental impacts is warranted. Thus, we synthesized copper sulfide nanoparticles (CuS NPs) to investigate whether the in-house synthesized Cu-based nanomaterials could inhibit the disease progress of *Gibberella fujikuroi*-infected in rice. The results showed that CuS NPs reduced the disease incidence on rice by contact-mortality, nutritional modulation, and phytohormone regulation. Our study provides important information for developing efficient delivery of nano-enabled agrichemicals and understanding the defensive pathways triggered by the nanoscale agrichemical use on crops.

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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Abstract

The use of nanotechnology to suppress crop diseases is gaining increasing interest in agriculture. Copper sulfide nanoparticles (CuS NPs) were synthesized at 1:1 and 1:4

ratios of Cu and S and their respective antifungal efficacy was evaluated against the pathogenic activity of *Gibberella fujikuroi* (Bakanae disease) in rice (*Oryza sativa*). In a 2-d *in vitro* study, CuS (1:1) and CuS (1:4) NPs at 50 mg/L decreased *G. fujikuroi* Colony-Forming Units (CFU) by 35.7 and 33%, respectively, compared to controls; commercial CuO NPs caused an 18.7% inhibition. In a greenhouse study, treating with both types of CuS NPs at 50 mg/L at the seed stage significantly decreased disease incidence on rice by 35.1 and 45.9%, respectively. Comparatively, CuO NPs achieved only 8.1% disease reduction, and the commercial Cu-based pesticide Kocide 3000 had no impact on disease. Foliar-applied CuO NPs and CuS (1:1) NPs decreased disease incidence by 30.0 and 32.5%, respectively, which outperformed CuS (1:4) NPs (15%) and Kocide 3000 (12.5%). Notably, CuS (1:4) NPs also modulated the shoot salicylic acid (SA) and Jasmonic acid (JA) production to enhance the plant defense mechanisms against *G. fujikuroi* infection. These findings provide useful information for improving the delivery efficiency of agrichemicals via nano-enabled strategies while minimizing their environmental impact, and advance our understanding of the defense mechanisms triggered by the NPs presence in plants.

Keywords: Cu-based NPs, CuS NPs, *Gibberella fujikuroi*, *Oryza sativa*, dissolution, phytohormones

1. Introduction

Copper (Cu)-based pesticides have been widely used as a broad-spectrum management strategy in agriculture over the past century. One of the most commonly used Cu-based pesticides on the market is a fungicide called Kocide, which has been extensively used on vines, vegetables and field crops, with maximum seasonal dose recommended at 7.1-160 pounds/acre.[1] Although such conventional Cu-based pesticides have shown efficacy to suppress a range of crop diseases, [2] extensive application over time has led to significant Cu contamination of soils. It has been reported that Cu contents in vineyard soils can reach 34-700 mg/kg in European vineyards due to frequent application of conventional Cu fungicides.[3] Such high Cu accumulation in soils after years of continual use can result in toxicity to non-target organisms, contamination of fresh water sources, and potential risks to human health. For example, toxic effects have been observed on tomato and barley grown in Cu-spiked soil, with ED50 value of 190-828 mg/kg and 240-937 mg/kg for tomato growth and barley root elongation, repectively.[4] Furthermore, Cu is transported from soil to adjacent surfaces waters. Bereswill et al. reported the in-stream water near a vineyard contained 0-67.6 μg/L total dissolved copper; in the sediment phase, the concentration was in a range of 7.3-116.9 mg/kg, which is 10 times higher than the geological background concentration (1.6–6.7 mg/kg dw).[5] Additionally, although Cu is essential for human health, rapid gastric disease has been noted upon exposure to Cu at 10 mg/L.[6] Thus, a novel and sustainable strategy for improving the antimicrobial efficiency of pesticides while simultaneously minimizing their negative impacts on ecosystems is needed.

There has been increasing interest in the use of nano-enabled approaches in agriculture, largely due to the unique properties and functions of materials at the nanoscale (e.g. greater activity, availability and tunability). A number of studies have demonstrated that Cu-based NPs exhibit significantly greater antimicrobial activity *in vitro* and relatively higher delivery efficiency for plant disease suppression and enhanced nutritional content as compared to conventional materials.[7–9] For example, Malandrakis et al. investigated the sensitivity of seven fungal species to metal-based NPs exposure *in vitro*, including Cu, CuO, Ag and ZnO; Cu NPs had the greatest mycelial growth inhibition rate, with a mean effective concentration (EC_{50}) at 307 μg/mL.[10] In a greenhouse study, Chen et al. reported that root exposure of CuO NPs at 50 mg/L significantly reduced the disease occurrence of soilborne *Ralstonia solanacearum* in infected tobacco by 22.3% relative to diseased controls, although no comparison with traditional Cu treatment was included.[11] Similarly, Elmer and White demonstrated that foliar application of CuO NP-solution onto *Fusarium oxysporum*-infected tomato and *Verticillium dahlia*-infected eggplant increased the crop yield by 33% and 34%, respectively, relative to the respective untreated infested controls.[12]

Most work investigating the antimicrobial activity of Cu-based NPs have focused on the impact of dose or other chemical properties of the metal oxide, such as particle size, solubility, and stability.[2,9] Few studies have looked at alternative metal species of Cu NPs; for example, sulfur (S) is known to be involved in abiotic and biotic stress response, as well as in secondary metabolism. Sulfur assimilation and its relationship with phytohormones, which are involved in plant pathogen defense pathways, have been studied extensively.[13] Sidhu et al. reported that synthesized copper sulfide (CuS) NPs showed antifungal activity *in vitro*, but no other Cu particles were used as a comparison.[14] Importantly, the ability of CuS NPs to suppress plant disease, increase nutrient uptake, and induce key defensive pathways (e.g. phytohormones) is largely unknown.

In the current study, we hypothesized that the smaller particle size and higher dissolution rates of CuS NPs compared to CuO NPs or Kocide can provide more effective disease suppression in pathogen-infected rice. We synthesized CuS NPs by altering the molar ratios of CuO NPs and Na_2S at 1:1 and 1:4 and evaluated antifungal efficacy against *Gibberella fujikuroi* with *in vitro* and *in vivo* studies with rice. Cu-based particles were applied as either seed treatment or as foliar-spray to *G. fujikuroi* infected rice seedlings. Plant growth, disease progress, nutrient uptake and defense-related phytohormone production were measured. These findings provide important information for precise and efficient delivery of nano-enabled agrichemicals and further our mechanistic understanding of the defensive pathways triggered by the nanoscale agrichemical use on crop species.

2. Materials and Methods

2.1 *In vitro* study

The protocol for CuS NP synthesis is described in Li et al.;[15] the details on synthesis and characterization (transmission electron microscopy, X–ray diffraction and initial hydrodynamic diameter) are presented in **Experiment S1 and Figure S1**. In order to test the antifungal activity of CuS NPs, a conidial suspension of *G. fujikuroi* was prepared as 100 conidia per milliliter (collection method is provided in **Experiment S2**) and was exposed to CuS NPs, and commercial CuO NPs and Kocide 3000 at 50 mg/L Cu (50 mg/L was selected as an optimum dose for comparison; descriptions are shown in **Experiment S3, Figure S2**) in Potato Dextrose Broth (PDB) medium at 25 °C for 12, 24, 48, 72, and 168 h. To uniformly disperse NP suspension into PDB, we directly added the freshly prepared Cu-based NP suspensions into PDB medium after dispersion using an ultra-sonicating probe. The mixture was shaken at 110 rpm for 10 min and then the welldispersed suspensions were obtained. A plate count method was used to quantify the Colony-Forming Units (CFU) of *G. fujikuroi* at different time points under the various treatments. In addition, dehydrogenase activity was used to estimate *G. fujikuroi* activity upon exposure to CuO and CuS NPs; the details of method are presented in **Experiment S4**. The dissolution rate of 50 mg/L Cu-based particles was evaluated in nanopure water (Nanopure ultrapure water system, Barnstead, IA, USA) and PDB with conidia; the details on the determination of dissolution rate are provided in **Experiment S5**.

2.2 Greenhouse study

Rice seeds (*Oryza sativa* L.) were surface sterilized with 30% (*v/v*) H_2O_2 for 10 min and infected by exposing to conidial *G. fujikuroi* suspension for 24 h; detailed procedures of infection are described in **Experiment S6**. The infected rice seeds or seedlings were treated with 50 mg/L Cu-based NPs via seed exposure or foliar application. Seed germination suggested that 15 µg Cu/seed was the optimum concentration for additional investigations (**Experiment S7, Figure S3**). There were six replicates (20 seeds per replicate) in each treatment. For seed treatment, 20 infected seeds were soaked into 6 mL of 50 mg/L Cu-based NP suspensions (equivalent to 15 µg Cu/seed) or sterile nanopure water and shaken at 110 rpm for 24 h at room temperature. All NP-treated seeds were germinated in autoclaved vermiculite and grown under greenhouse conditions for 5 weeks. For foliar application, the infected seeds were transferred into autoclaved vermiculite directly without NP exposure. After two weeks, the infected seedlings were foliar-treated with 50 mg/L NP-suspension or sterile nanopure water as diseased control. The NP-foliar application was applied twice within a 7-day interval with an actual volume transferred onto seedling of each replicate was 0.5 mL. The plants were then grown for 21 days. Non-infected (healthy) and non-NP treated seedlings (diseased control) seedlings were set as separate controls. The greenhouse condition and nutrient supply for all plants are shown in **Experiment S7**. After growing in the vermiculite for 5 weeks, rice seedlings were harvested, and disease incidence was evaluated based on the symptoms progress; the details of evaluation are in **Experiment S8**.

2.3 Plant Nutrient and pigment analysis

Four hundreds milligrams of plant tissues (shoot/root) were digested with concentrated nitric acid (5 mL) and hydrogen peroxide (1 mL).[16] and the content of K, Ca, Cu, Fe, Mg, Mn, P, S, and Zn was quantified using inductively coupled plasma optical emission spectroscopy (ICP-OES, iCAP 6500, Thermo Fisher Scientific, Waltham, MA). In addition, chlorophyll and total phenolics contents were measured by the method of Lichtenthaler and Folin-Ciocalteu assay, with minor modifications (Details are in **Experiment S9 and S10**).

2.4 Phytohormone and phytoalexin measurement

Rice tissues were ground in liquid nitrogen with a mortar and pestle, and then 400 mg of the ground tissue were transferred into a 15 mL centrifuge tube containing 4 mL extraction solution consisting of a mixture of 2-propanol/H_2O/ concentrated HCl (2:1:0.002, *v/v/v*). All samples were vortexed for 1 min and then shaken at 120 rpm at

4 °C for 30 min. Four mL of dichloromethane were then added, and the sample was shaken for another 30 min at 4 °C. The samples were centrifuged at 5000 rpm and 4 °C for 10 min, and the bottom layer of solution was collected and concentrated under nitrogen. The concentrated samples were re-dissolved in 0.7 mL of CH_3OH/H_2O (8:2; *v/v*) for hormone and phytoalexin measurement. The phytohormone and phytoalexin content were measured by high performance liquid chromatography (HPLC; UV-Vis detector model: SPD-M20A; SHIMADZU, Kyoto, Japan). The column (C6-Phenyl 110A, 250 × 4.60 mm 5 micron, phenomenex, Torrance, USA) temperature was set at 25 °C. Solvent A was H_2O with 0.1% HCOOH, and solvent B was CH_3CN. Elution was programmed as follows: 20% solvent B for 15 min, followed by a linear gradient from 20% to 100% B in 20 min, and finally holding at 100% B for 5 min. The quality assurance/quality control (QA/QC) information is shown in **Experiment S11**.

2.5 Statistical analysis

The results from all the experiments are expressed as the mean ± standard error. A parametric one-way analysis of variance (ANOVA) followed by a Fisher LSD test was conducted ($p<0.05$). All the analyses were performed using Excel 2010 and OriginPro 2019b (Originlab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1 *In vitro* toxicity

CuS (1:1) NPs at 50 mg/L Cu decreased the CFU by 35.7 and 20.8% and CuS (1:4) NPs decreased the CFU by 33 and 17.6%, relative to the control and CuO NPs, respectively at 48 h. Similar trends were evident at 72 hours. It is worth noting that the antifungal activity of CuO NPs was significantly less than both types of CuS NPs in the first 72 hours, but that pattern was reversed at 168 hours (**Figure 1A and B**), at which the CFU of *G. fujikuroi* in both CuS NP treatments was equivalent to the untreated control. It is noted that Kocide 3000 exhibited the highest antifungal efficiency during the experiment. In addition, the pattern of dehydrogenase activity of *G. fujikuroi* at 168 hours across all treatments aligned with the fungal growth data (**Figure 1C**).

Cu-based NPs are known to reduce microbial growth by direct contact between cells and NPs/Cu ions.[17] It has been proposed that NPs/ions can attach to cell membranes and disrupt membrane potential and membrane-related electron transfer system, resulting in leakage of electrolytes and eventually leading to the cell lysis and death.[2] In addition, interaction between cellular components and NPs/dissolved Cu ions can cause DNA damage and protein inactivation, which is considered to be another antimicrobial mechanism of NPs.[18,19] It is noted that specific properties of NPs such as particle size, shape, and composition can greatly affect antimicrobial activity; in general, dissolution rate most often plays a dominant role in microbial control. For instance, Borgatta et al. highlighted that synthesized $Cu_3(PO_4)_2 \cdot 3H_2O$ nanosheets exhibited a significantly greater dissolution rate than CuO NPs and that this release profile correlated with a higher inhibition rate of Fusarium wilt growth in watermelon.[20] Therefore, we measured the release Cu ions from each type of NPs to fully understand the mechanisms of antifungal activity of different Cu-based NPs, as well as the observed differences as a function of particle type.

3.2 Cu dissolution

Over 7 days, the dissolution rates of ionic Cu from CuS (1:1) and CuS (1:4) NPs were increased from 1.77% to 9.48% and from 2.89% to 7.82%, respectively, which were several-fold higher compared to the CuO NPs (**Figure 1D**). For Kocide 3000, release of Cu occurred rapidly in the first 12 hours but was stable at approximately 5% for the duration of the experiment. The Cu dissolution kinetics are quite different in the PDB medium. As shown in **Figure 1E**, both types of CuS NPs displayed significantly greater dissolution (14 and 15%) in PDB than did CuO NPs (10%) in the first 48 hours. However, at 72 hours, Cu release from the CuO NPs had increased to 35%, which was two-folder higher than the CuS (1:1 and 1:4) NPs (15% and 16%). This reversed pattern continued to 168 hours, at which 69.3% of total CuO NPs was dissolved into Cu ions, which was more than 3-fold higher than CuS (1:1 and 1:4) NPs (16%). The patterns of Cu-based NP dissolution in two different media were largely dependent on the pH and composition of the medium. Numerous studies demonstrated that pH and dissolved organic matter (DOM) increased the level of dissolved Cu species from Cu-based NPs. For instance, Gao et al. reported that decreasing the soil pH from 6.8 to 5.9 increased the dissolution rate constant from 0.17 $mol^{1/3}\cdot kg^{1/3}\cdot s^{-1}$ to 0.56 $mol^{1/3}\cdot kg^{1/3}\cdot s^{-1}$.[21] Liu et al. found that at dissolved humic acid (DHA) concentration of 57.07 mg $C\cdot L^{-1}$, the total amount of Cu^{2+} released from CuO NPs during 72 h was more than 13 times higher than that in the absence of DHA.[22] In this study, the pH in H_2O and PDB medium was kept at 5.6 and 5.2, respectively, over 7 days. In addition, one of the major components in PBD is dextrose, which has been reported to complex with Cu ions.[23] Thus, the lower pH of PDB than nanopure and the presence of DOM made the Cu dissolution from CuO NPs more thermodynamically favorable, by a proton-promoted process and/or a ligand-assisted dissolution process.[24] In addition to pH and DOM, the redox condition in the medium is another important factor to determine the dissolution of Cu-based NPs. The redox potential in the actual environment for fungal growth (such as the rhizosphere in rice) might vary due to water management. Under oxidation conditions, synthesized CuS NPs (1:1 and 1:4) could be more reactive because of the presence of S^{2-}. The sulfide could be oxidized, and the conversion to oxidized sulfur species might destabilize CuS and lead to higher Cu dissolution rate.[25]

Overall, the significant release of Cu ions determined the reactivity of Cu-based NPs, and played a critical role in the observed antifungal activity. Free Cu ions are known to be highly redox-active species and can generate excessive amounts of hydroxyl radical by Fenton-like reactions.[25] Reactive oxygen species ($\cdot HO_2$ and $\cdot HO$) can lead to cellular oxidative stress and damage to multiple cellular organelles and biomolecules.[26] In the current study, the smaller initial particle size (approximately 5-10 nm) and hydrodynamic diameter (249±0.5 and 252±0.18 nm) of CuS (1:1 and 1:4) NPs (**Figure S1**) resulted in greater Cu dissolution in PDB and subsequently inhibited the fungal growth during the first 48 hours. Beyond 48 hours, the higher solubility of CuO NPs and Kocide 3000 could more effectively control the *G. fujikuroi* growth in PDB. However, the above *in vitro* results in PDB were different from the rice grown in a solid medium. To mimic the more realistic conditions, instead of PDB, Cu-based NP suspension was prepared in nanopure water and applied to *G. fujikuroi*-infected rice. Not surprisingly, it was found that the CuS NPs (1:1 and 1:4) with a higher amount of dissolved Cu ions in nanopure water outperformed Kocide 3000 in terms of antifungal efficiency (discussed below in the next section).

3.3 Greenhouse study

Disease incidence Rice seedling growth was significantly inhibited with infection by *G. fujikuroi* (**Figure 2A and E**), with a 60% reduction of fresh shoot biomass in the diseased treatment (0.38g) as compared to the healthy control (0.94g). In the seed treatment, nanoscale CuO NPs, CuS (1:1) and CuS (1:4) significantly decreased disease incidence by 8.1%, 35.1, 45.9%, respectively, compared to the diseased control (**Figure 2B**). The findings demonstrate that the three types of Cu-based NPs significantly inhibited invasion by *G. fujikuroi*, although the two sulfide forms were most effective. Kocide 3000 at the same concentration of Cu did not impact disease incidence (**Figure 2B**), although we must note that this formulation is not intended for use as a seed treatment. Regarding foliar application, CuO NPs and CuS (1:1) NPs significantly decreased disease incidence to the greatest extent (30% and 32.5%); CuS (1:4) NPs and Kocide 3000 were less effective but still significantly reduced disease incidence by 15% and 12.5%, respectively (**Figure 2B**). These findings suggest that both seed treatment and foliar application of 50 mg/L Cu-based NPs can significantly reduce the severity of bakanae disease, with efficacy being significantly greater than the commercially available Kocide 3000. It has been reported that CuO and Cu_2O NPs were more effective against *Phytophthora infestans* on tomatoes than registered commercial Cu products.[7] Similarly, foliar exposure of CuO NPs to tomato and eggplant grown under greenhouse and field conditions reduced the disease progress of *Fusarium* wilt fungus and increased the fruit yield.[12]

The results from the greenhouse study suggest that CuS NPs (1:1 and 1:4) performed better in disease suppression as compared to Kocide 3000, although the *in vitro* study indicated that the latter displayed the best antifungal efficacy. The reason for the different antifungal patterns of Cu-based NPs and Kocide 3000 between *in vitro* and *in vivo* studies may be due to their different particle sizes and Cu release rates. The CuS (1:1 and 1:4) NPs with smaller size and higher amount of dissolved Cu in NP-suspension (in nanopure water) showed better antifungal performance (**Figure 1D**) relative to Kocide 3000. Our findings are also supported by the work of Borgatta et al., in which the customized $Cu_3(PO_4)_2 \cdot 3H_2O$ nanosheets with greater Cu dissolution had significantly greater inhibition of Fusarium in watermelon than the commercial CuO NPs as measured by yield and disease progression.[20] In addition, as an important plant nutrient, sulfur (S), known to be involved in the secondary metabolism and stress tolerance, can contribute to suppressing disease.[13] Thus, our findings suggest that both physical and chemical properties of NPs are important to their antimicrobial activity and that nanomaterials could be tuned to have specifically desired release and impacts on biota.

Chlorophyll content and total phenolics Chlorosis is a common symptom of disease in *G. fujikuroi* infected plants. In the current study, the total chlorophyll content in diseased controls was significantly reduced by 42.8 and 48.8% compared to respective healthy controls. In the seed treatment, the total chlorophyll content in the CuO, CuS (1:1) and CuS (1:4) NPs treated rice seedings was increased by 72.4, 68.8 and 46.1%, respectively (**Figure 2C**), as compared to the diseased control. Notably, these values are similar to the healthy control. Foliar exposure of Cu-based NPs to *G. fujikuroi* infected rice seedlings showed the similar pattern of total chlorophyll content in rice, with the greatest increase (58.7%) upon exposure to CuO NPs. Interestingly, the magnitude of enhancement from other foliar nanoscale treatments was less than that with the seed treatment (**Figure 2C)**.

Phenolic compounds are involved in biotic stress-induced defense systems in plants.[27] The total phenolic content in the diseased control increased by 19.8 and 31.6% in the seed treatment and foliar application, respectively, when compared to healthy control (**Figure 2D**). Importantly, both the seed/foliar treatment with Cu-based NPs significantly reduced the total phenolic content to that of the healthy control level. Awan et al. reported a 25-80% increase in the phenolic content in a range of tomato resistance genotypes upon infection with *Alternaria solani* .[28] Similarly, increases in the total phenolic content were reported in lettuce upon infection with *Rhizoglomus irregulare* and that this increase in phenolics was positively correlated with antioxidant enzyme activity.[29] In summary, the addition of Cu-based NPs reduced the total phenolic level of infected plants to that of disease free controls, indirectly demonstrating that Cu-based NPs convey tolerance to fungal disease in rice seedlings.

Nutritional content Both seed and foliar treatments resulted in different Cu uptake and accumulation patterns in *G. fujikuroi*-infected rice seedlings. In the seed treatment, Cu exposure did not significantly change the shoot Cu content compared to the healthy and diseased control (**Figure 3A**); however, CuO, CuS (1:1) and CuS (1:1) NPs significantly increased the root Cu content by 25.7, 36.8 and 62.9%, respectively. Importantly, the CuS (1:4) NPs delivered more Cu to the roots than the other particles (**Figure 3B**). Regarding foliar treatment, not surprisingly, significantly greater Cu levels were found in all nanoscale treatments (**Figure 3A and B**). Interestingly, both types of CuS NPs transferred significantly more Cu to the roots, with more than 36.4% greater Cu level than CuO NP treatment (**Figure 3B**), although no significant difference was evident between CuS NPs and Kocide 3000. We suggested that the smaller particle size and higher Cu dissolution rate of CuS NPs (1:1 and 1:4) relative to CuO NPs contributed to the higher Cu translocation rate. The root content of Cu and other micronutrients is known to be a critical component of plant resistance to fungal infection[17,31]. For example, foliar application of CuO and $Cu_3(PO_4)_2$ at 50 mg/L increased watermelon shoot Cu content as compared to diseased control.[20] In the current study, the increased levels of Cu in NP-treated rice seedlings played a crucial role in suppressing Bakanae disease. Given the *in vitro* data presented above, we hypothesize that Cu could suppress the pathogen directly by contact-killing. In addition, Cu is an essential nutrient involved in plant defense and secondary metabolism and as such, may improve tolerance to fungal infection.[12]

Infection with *G. fujikuroi* significantly decreased the S content in both rice shoots and roots relative to the respective healthy control (**Figure 3C and D**). There was no difference in the shoot S content as a function of either nanoscale material exposure route or material type (**Figure 3C**). In roots, the addition of Cu-based NPs at both the seed stage or foliar application increased the S content as compared to the diseased control by more than 42.9%, although no difference was found among different particle types (**Figure 3D**).

The content of essential macronutrients in rice shoots and roots across all the treatments are shown in **Table 1**. Root infection of *G. fujikuroi* significantly decreased K and P content by 8.42-16.67% in shoots and K, P and Mg content by 14-66% in the roots relative to healthy controls. Similarly, Singh et al. demonstrated that *G. fujikuroi* could infect rice seedlings via roots and the base of stem, and accumulate in vascular bundles.[30] Thus, decreases in the nutritional content of rice shoots and roots could be attributed to the disease-induced inhibition of water and nutrient transport. However, upon Cu treatment, the K content in shoots and roots was significantly increased compared to the diseased control. In particular, seed application of CuO NPs, CuS (1:1) NPs, and CuS (1:4) NPs increased the root K level by 124, 148 and 144%, respectively; the increased K level achieved with the Kocide 3000 treatment was less than 50% of that treated with Cu-based NPs. Similarly, foliar application of CuS (1:1) NPs, CuS (1:4) NPs and Kocide 3000 significantly increased the root Ca content by 23.4, 21.8 and 11.8%, respectively, as compared to the diseased control. In addition, both seed and foliar applications of Cu-based particles increased the root P content by 10.7-74.8% relative to diseased control. Last, both exposure routes of Cu-based NPs significantly increased root Mg contents from 61.1-155% when compared to the diseased control, although no differences were evident in shoots across all the treatments.

The micronutrient content in rice shoots and roots is shown in **Table 1**. Infection by *G. fujikuroi* significantly decreased root Fe and Mn content by more than 30% in comparison with healthy controls. Upon seed- and foliar-exposure to Cu-based NPs, the root Fe content was significantly increased as compared to the diseased control, with the highest increase with foliar-CuS (1:1) and CuS (1:4) NP treatments (210 and 198%); Similarly, in comparison with the diseased control, a 48.3% increase in the Zn content were found in roots foliar-treated with CuS (1:4) NPs. Foliar application of both CuS (1:1) NPs and CuS (1:4) NPs increased the root Mn content by 61.5 and 50.9%, respectively; for the seed treatment, CuS (1:1) NPs increased the root Mn content 83.3%.

Plant macro- and micronutrients play important roles in suppressing plant disease. For example, the application of K fertilizer has been shown to activate phenylalanine ammonia lyase and polyphenol oxidase in soybean, subsequently suppressing soybean cyst nematode, a severe soil borne disease.[31] Ca is a component of secondary messenger molecules and could activate and improve plant defense systems to plant pathogens. Debona et al. found that exposure to 5 mM Ca increased the expression levels of genes in the salicylic acid and jasmonic acid pathways in *Pyricularia oryzae*-infected wheat as compared to those treated with 0.26 mM Ca.[32] Fe has been shown to alter nonenzymatic antioxidant potential in watermelon seedlings, as well as to induce jasmonate-linked defense responses.[33] The function of Zn in plant protection is related to superoxide dismutase and zinc finger proteins.[34] Mn plays important roles in enhancing pathogen-induced lignification, callus formation, ROS production and in reducing pathogen-induced cell death.[35] Therefore, appropriate or even elevated tissue concentration of these elements as a function of seed or foliar nanoscale treatment can protect plants against pests and diseases by stimulating plant immune response.[36]

Phytohormone content Phytohormones are known to be a critical component of plant defense and response to biotic stress.[37–39] Jasmonic acid (JA) is associated with triggering plant defense against herbivores and necrotrophic pathogens.[40] The JA content in the diseased control was 50% less than that of the healthy control (**Figure 3E**), suggesting that *G. fujikuroi* infection might suppress the JA synthesis in rice shoots.

Similarly, Siciliano et al. demonstrated that the JA level in *G. fujikuroi* infected rice was reduced.[40] Navarro et al. demonstrated that the increases in gibberellic acid induced by *G. fujikuroi* effectively suppressed the JA signaling.[41]

Foliar application of different types of Cu did not increase the JA content in the infected shoots. However, in the seed treatment, CuS (1:4) NPs significantly increased *in planta* JA content to levels equivalent to the healthy control level (**Figure 3E**). Wang et al. reported the incidence of gray mold disease in JA-deficient mutant tomato was higher than that in the corresponding wild-type, suggesting the important role of JA in disease suppression.[42] In addition, exogenous addition of JA increased the production of NO and H_2O_2, as well as the activity of antioxidant enzymes.[42] Many previous studies demonstrated that JA increased the expression of defense-related genes.[43] Our findings relative to JA in the seed treatment with CuS (1:4) NP suggest that stimulating this phytohormone may amplify pathogen-induced defense systems and subsequently reduce the incidence of bakanae disease in rice.

Abscisic acid (ABA) is an indicator of abiotic stresses such as salinity, drought and cold, and has been shown to regulate plant defense responses.[44] In the seed treatment, Cu-based NPs significantly elevated the shoot ABA content as compared to the diseased control; foliar application had a similar increasing trend for ABA content in *G. fujikuroi* infected shoots (**Figure 3F**), although no difference was evident as a function of particle types. Our results suggest that Cu-based NPs increased the ABA level in rice and further triggered innate defense systems. Also, upon exposure to 20 and 100 mg/L ZnO NPs, the ABA level in *Arabidopsis thaliana* was significantly increased.[45] Exposure to 1000 mg/L CuO NPs increased the ABA content in cotton,[46] and Kaur et al. demonstrated that rice resistance to *G. fujikuroi* was significantly increased by ABA.[47] Likewise, rice basal resistance against the brown spot caused by *Cochliobolus miyabeanus* was enhanced by exogenous application of ABA; the authors speculated that the positive role of ABA in resistance could be affected through antagonistic crosstalk with the ethylene signaling pathway.[48]

Activation of salicylic acid (SA) biosynthesis and signaling pathways can lead to reactive oxygen species and pathogenesis-related protein accumulation, and induction of the systemic acquired resistance and hypersensitive responses.[49] However, endogenous SA levels vary among different plant species.[50] No difference of the SA content was evident across all Cu-based NP treatments when compared to the healthy and diseased control (**Figure 3G**). In agreement with our results, Iwai et al. reported that the SA content in rice was stable under pathogen attack, especially at seedling stage.[51] Conversely, high levels of SA accumulated in *Arabidopsis* upon bacteria pathogen *Pseudomonas syringae* infection.[52] In our current study, the high level (12.7-14.5 μg/g) of SA in rice might serve as ROS scavenger to protect plants from oxidative damage.

Sakuranetin (SN) is one of most important phytoalexins in rice to defense against infection.[40] *G. fujikuroi* infection significantly reduced the shoot SN content; a 37.7% reduction in the SN content was found as compared to the healthy control (**Figure 3H**). Foliar exposure to all types of Cu-based particles significantly increased the SN content as compared to infected control (**Figure 3H**). In particular, exposure to CuS (1:4) NPs increased the SN level by 96.4% relative to the diseased control. For the seed treatment, CuO NPs significantly increased the SN content over the diseased control, although no significant changes were evident for the other Cu treatments. Cu-induced SN production can contribute positively to defense-related systems in plants.[53] Sudo et al. reported that defense-related genes in $CuCl_2$ treated rice leaves were strikingly up-regulated; in particular, genes involved in phytoalexin and lignin biosynthesis pathways were sensitive to Cu.[54] Similarly, Cu was an abiotic elicitor to induce the SN accumulation in rice leaves.[55] In the current study, nanoscale Cu may have triggered the phytoalexin biosynthesis, which played an important role in suppressing the *G. fujikuroi* infection.

Phytohormone crosstalk provides the plant with a powerful capacity to regulate immune responses.[56] An antagonistic crosstalk between JA and SA has been widely reported; the former is needed for resistance to necrotrophic pathogens, the latter can respond to biotrophic pathogens-induced biotic stresses.[57] For example, Spoel et al. demonstrated that upon the infection by biotrophic *Pseudomonas syringae*, tobacco activated SA-associated defense systems and simultaneously suppressed the JA signaling pathway.[58] On the contrary, in our study, the SA content in the diseased control was stable; while the JA content was increased in CuS (1:4) NPs treated rice relative to the diseased control. Thus, exposure to CuS (1:4) NPs could modulate the crosstalk between SA and JA as needed as part of the suppression of the necrotrophic Bakanae disease.

ABA is known to balance SA and JA pathways and to subsequently mediate an appropriate immune response to invading pathogens.[59] In the present study, the increased ABA level in NP-treatments may have triggered the JA signaling pathway in response to fungal infection. As an abiotic effector, Cu-based NPs appears to be a positive promoter to help the plant defend against pathogen infection by regulating phytohormone levels. Hao et al. reported that nanomaterials increased plant resistance to fungal infection by altering the endogenous hormone content.[60] Taken together, our findings suggest that Cu-based NPs enabled crosstalk between JA, SA, and ABA; mediated plant response to *G. fujikuroi* infection; and effectively balanced the defense response while promoting plant growth.

4. Conclusion

Our synthesized CuS NPs exhibited great antifungal efficacy against *G. fujikuroi*. Smaller size/higher dissolved Cu from CuS (1:1 and 1:4) NPs enhanced their antimicrobial activity and use efficiency, and therefore, can greatly reduce the application level of active ingredient (i.e., Cu), which can lead to lower loading and pressure of Cu to the environment. Additionally, NP-induced production of phytohormones could exert an important role in enhancing the antimicrobial response, which could be considered as a novel strategy for disease suppression and crop growth enhancement. Overall, optimizing the delivery efficiency of Cu pesticides by tuning nanoscale properties such as size, charge, composition, morphology, and dissolution rate is a promising solution to maintain or increase crop production while minimizing negative environmental impacts. A better understanding on the mechanisms of antimicrobial activity of Cu-based NPs will significantly advance nano-enabled disease management strategies for sustainable agriculture.

Associated Content

Supporting information include eleven experiments and three figures.

Acknowledgement

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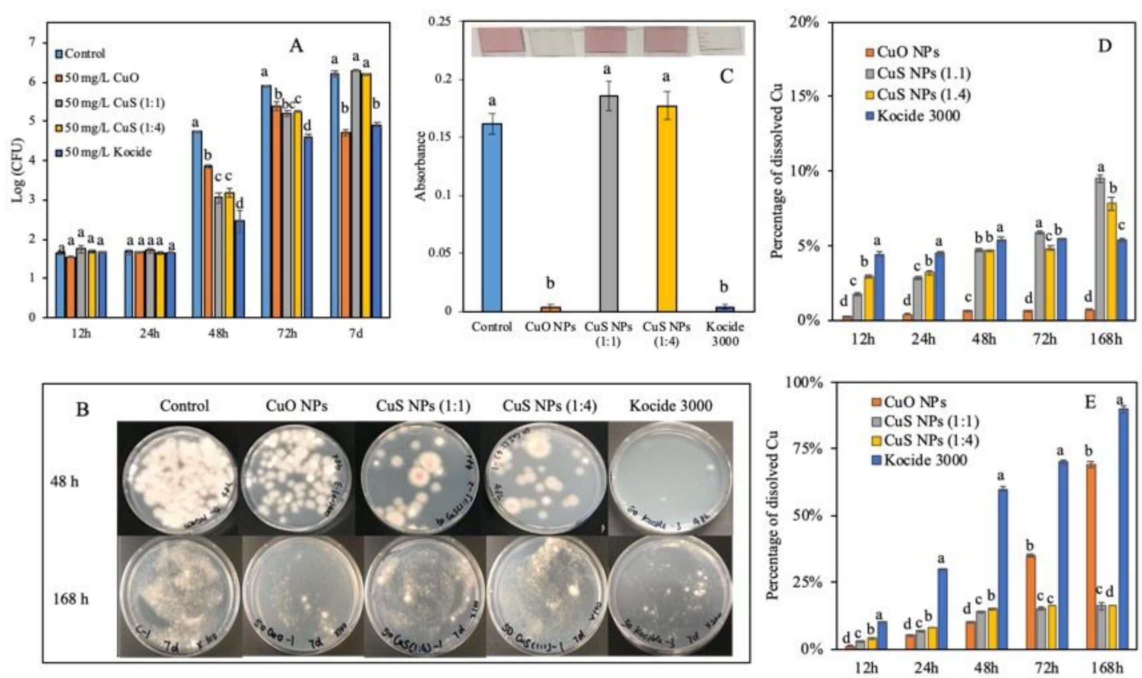

Figure 1. Growth inhibition of *G. fujikuroi* in PDB medium amended with Cu-based NPs at 50 mg/L and dissolution rate of Cu-based NPs at 50 mg/L over 168 hours. (A) Logarithm of CFU counts on petri dishes over 168 hours, (B) The growth of *G. fujikuroi* on petri dishes after treating with Cu-based NPs in PDB medium for 48 and 168 hours, (C) Dehydrogenase activity of *G. fujikuroi* after treating with CuO NPs in PBD medium for 168 hours, and dissolution rate of Cu-based NPs in (D) nanopure water and (E) PDB with conidia. The pH in nanopure water and PDB medium was kept at 5.2 and 5.6 respectively over 7 days. 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 $p < 0.05$.

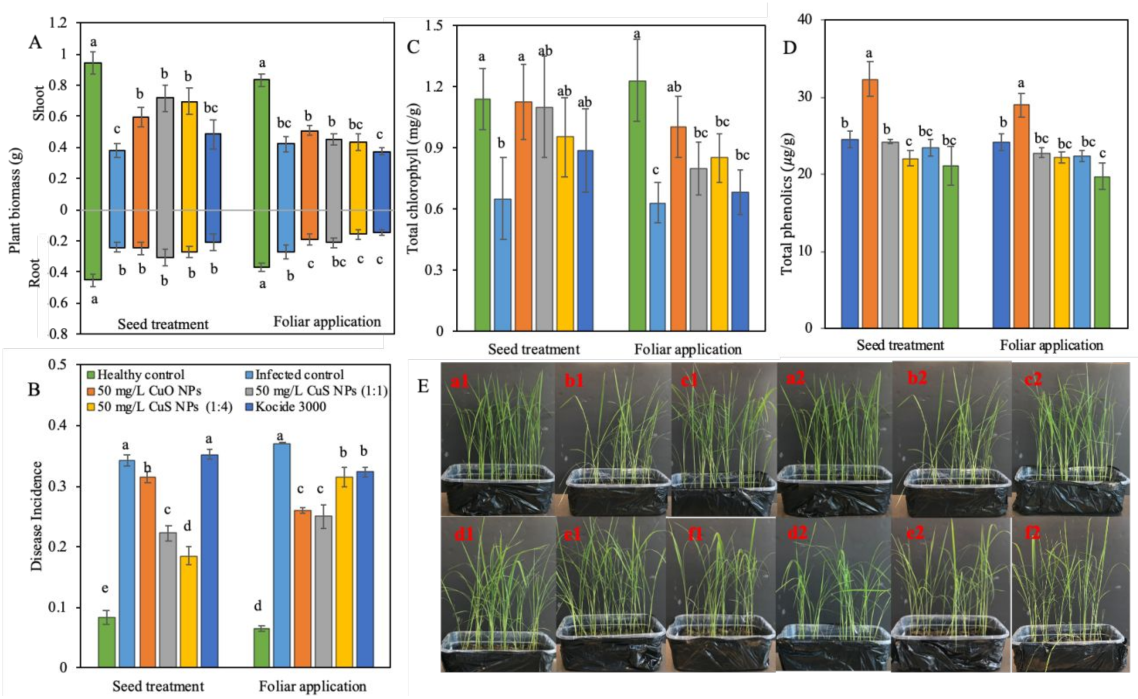

Figure 2. Physiological responses of *G. fujikuroi*-infected rice after exposure to Cu-based NPs. (A) Fresh shoot and root weight, (B) Disease incidence, (C) Total chlorophyll, (D) Total phenolic acid, and (E) phenotypic images of rice seedlings across all the treatments, including (a1-f1) Seed treatment (a2-f2) Foliar application (a, b, c, d, e and f represent for healthy control, Infected control, CuO NPs, CuS NPs (1:1), CuS NPs (1:4), and Kocide 3000, respectively). 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 $p < 0.05$.

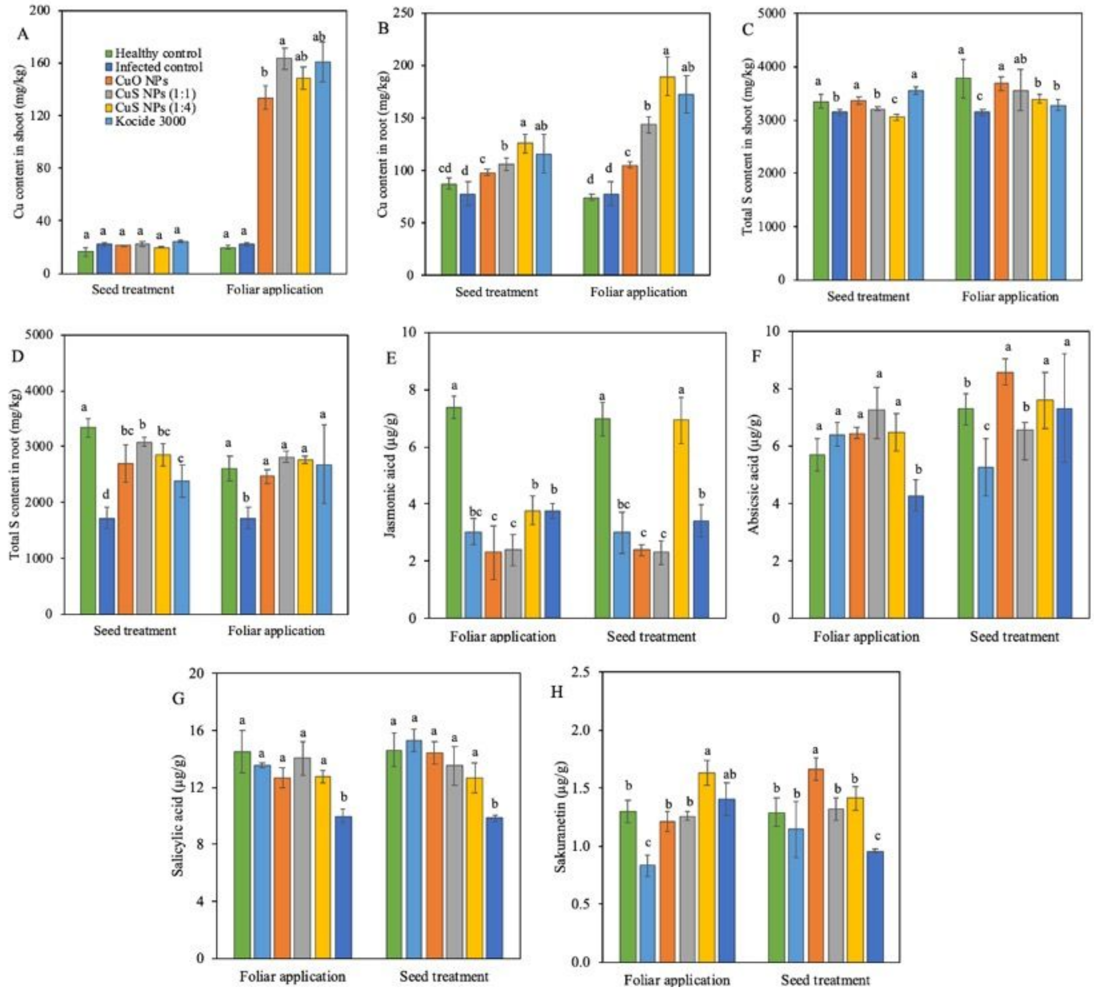

Figure 3. The content of Cu, S, and phytohormone in rice seedlings upon exposure to 50 mg/L Cu-based NPs. A) The Cu content in shoot, (B) The Cu content in root, (C) The S content in shoot and (D) The S content in root; (E) Jasmonic acid, (F) Abscisic acid, (G) Salicylic acid, and (H) Sakuranetin in shoot. 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 $p < 0.05$.

Table 1. The nutrient content in Cu-based NPs treated rice shoots and roots. 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 $p < 0.05$.

Route	Treatment	Nutrient content in rice shoots (mg/kg)						
		K	Ca	P	Fe	Mg	Zn	Mn
Seed treatment	Healthy control	31776±860b	2255±28a	3518±130b	145±26b	2832±52a	22±1a	663±2a
	Infected control	29101±340c	2131±160abc	3610±219ab	118±81b	2825±262a	25±3a	863±129a
	CuO NPs	35044±337a	2047±22c	3747±56a	234±47ab	2769±9a	22±3a	782±31a
	CuS NPs (1:1)	32591±1652b	2181±39b	3649±121ab	239±27a	2746±108a	23±1a	904±95a
	CuS NPs (1:4)	33179±1157b	1946±115c	3501±69b	162±24b	2641±245a	27±1a	807±26a
	Kocide 3000	30468±1990b	2183±23b	3342±78c	222±111ab	2895±155a	24±2a	792±72a
Foliar application	Healthy control	34199±2737ab	2220±235ab	4356±451a	130±12b	2887±276a	37±4a	829±37b
	Infected control	28497±141c	2124±101b	3723±89b	124±78b	2971±219a	24±3b	889±119b
	CuO NPs	36419±863a	2376±53a	4048±110a	213±26a	3131±152a	30±3b	1151±89a
	CuS NPs (1:1)	31210±3293b	2055±218b	3815±244ab	298±71a	3020±284a	28±1b	920±43b
	CuS NPs (1:4)	31829±1064b	2229±142ab	3774±156ab	245±9a	3010±118a	33±3b	955±65b
	Kocide 3000	32645±1281b	2008±117b	3552±123b	243±90a	2777±326a	29±3b	953±79b

Route	Treatment	Nutrient content in rice roots (mg/kg)						
		K	Ca	P	Fe	Mg	Zn	Mn

Seed treatment	Healthy control	20772±1027a	1376±39c	1966±88c	1469±328a	3657±187a	30±1a	173±16b
	Infected control	7755±1870c	1634±45b	1658±95d	894±133b	1588±140d	30±1a	118±14c
	CuO NPs	17400±3170ab	1650±35b	2412±255b	1923±483a	3314±597ab	27±2b	128±31c
	CuS NPs (1:1)	19277±934a	1839±271ab	2528±79a	1655±324a	3013±74b	25±0.5c	216±12a
	CuS NPs (1:4)	18931±1707a	1865±91a	2247±119a	1812±298a	3533±386a	29±2a	131±29c
	Kocide 3000	12532±2760b	1908±126a	1835±163ab	1448±219a	2560±337c	22±1d	97±4d
Foliar application	Healthy control	15464±1219a	1489±104c	1903±162c	1943±378a	4331±713a	34±4b	188±34a
	Infected control	6052±1067b	1644±35c	1634±85d	927±101b	1471±148c	29±1b	108±14b
	CuO NPs	14066±1052a	1543±86c	2477±24b	1937±195a	2884±352b	24±1c	137±14b
	CuS NPs (1:1)	16972±709a	2029±345ab	2857±220a	2878±1031a	4058±850ab	27±2c	184±13a
	CuS NPs (1:4)	15885±1055a	2002±39a	2799±114a	2684±1086a	3742±917ab	43±2a	173±16a
	Kocide 3000	15977±4768a	1838±77b	2686±647ab	1726±176a	3163±279b	32±7bc	193±75ab

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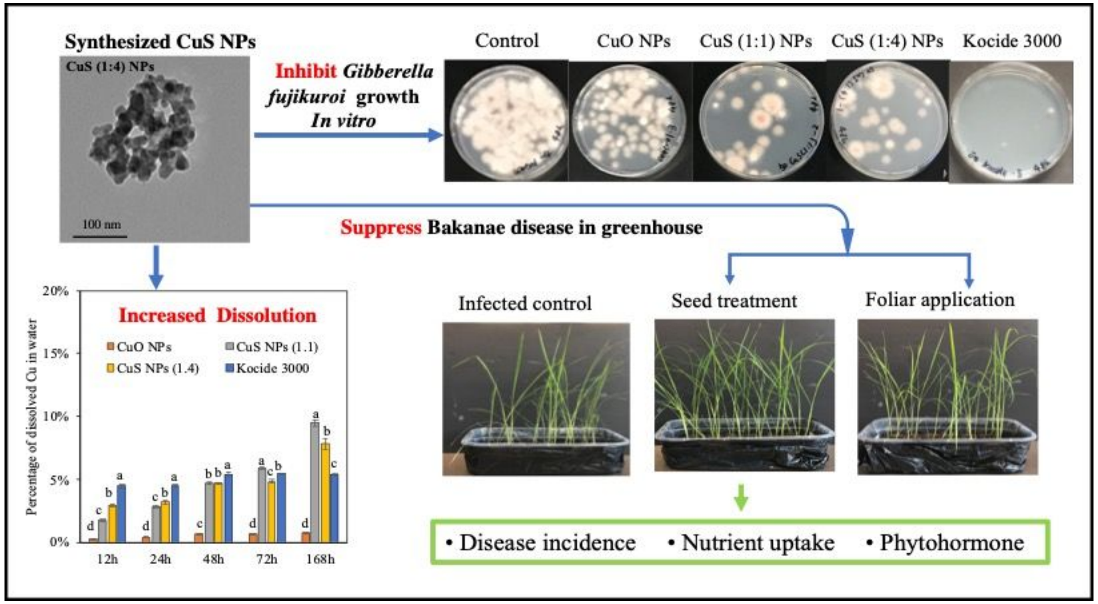
Synthesized CuS NPs
CuS (1:4) NPs
100 nm
Inhibit Gibberella fujikuroi growth In vitro
Control
CuO NPs
CuS (1:1) NPs
CuS (1:4) NPs
Kocide 3000
Suppress Bakanae disease in greenhouse
Increased Dissolution
CuO NPs
CuS NPs (1.1)
CuS NPs (1.4)
Kocide 3000
Percentage of dissolved Cu in water
20%
15%
10%
5%
0%
12h
24h
48h
72h
168h
Infected control
Seed treatment
Foliar application
• Disease incidence • Nutrient uptake • Phytohormone