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Mixed-Valence Core-Shell Copper Loaded Silica Nanoparticle – a Powerful Antimicrobial Composite Material for Agricultural Crop Protection

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Abstract -Antimicrobial activity of Copper (Cu) is known for centuries. Cu compounds are used in agriculture for protecting crops from bacterial and fungal diseases. Prolonged use of Cu as agricultural pesticides is detrimental to the environment due to undesired toxicity issues. In absence of suitable Cu alternative, an effort towards reducing the amount of Cu pesticide release in the environment is desirable. In this paper, we present a strategy to load Cu on the surface of a solid silica (inert) support with high surface area, thus forming a core-shell (C-S) Cu loaded silica particle (CuSiNP). It is hypothesized that Cu coated on an inert support will be still bioavailable and exhibit antimicrobial efficacy. In this design, the core volume of the Cu particle is replaced with an inert material (silica). Since Cu is contact killing pesticide, similar level of antimicrobial efficacy (per unit amount of metallic Cu) is expected from C-S CuSiNP material when compared with traditional Cu compounds. We have presented in this paper two different design of CuSiNPs. The silica core (inert) was synthesized using a modified Stöber sol-gel method. Cu SEM and TEM techniques were used to estimate particle size and morphology. Particle dispersibility in aqueous suspension was measured using Dynamic Light Scattering (DLS) technique. Antimicrobial efficacy was evaluated against *E. coli* (a gram negative bacteria) *B. subtilis* (a gram positive bacteria) using disc diffusion assay (DDA) and growth inhibition assay. Based on the bacterial growth inhibition (OD measurements), minimum inhibitory concentration (MIC) value of the CuSiNP material was estimated. Results showed that C-S CuSiNP materials possess improved efficacy in comparison to a commercial pesticide (Kocide®3000 fungicide/bactericide). Plant tissue injury potential (phytotoxicity) was evaluated using ornamental *Vinca sp*. as a model system. Results show that C-S CuSiNP material was non-phytotoxic.

***Keywords*:** Copper-Silica, Antimicrobial, Mixed-Valence, Core-shell, Nanoparticle, Pesticides

1. Introduction

Copper (Cu) is known as one of the oldest antimicrobial therapeutic agent in history (Borkow and Gabbay 2005). Cu compounds are used as wood preservative (Appendini and Hotchkiss 2002) and antifouling agents in paint based materials (Terlizzi, Fraschetti et al. 2001). In food packaging(Schultz, Nicholas et al. 2007, Freeman and McIntyre 2008) industry, Cu compounds are used as antimicrobial agent. In healthcare facilities, Cu based alloy materials are used to provide touch-safe (microorganism-free) surfaces such as door knobs and bed rails (Gant, Wren et al. 2007, Casey, Adams et al. 2010). Copper antimicrobial compounds are known to generate reactive oxygen species (ROS) which damages cell membrane through lipid peroxidation and actively take part in DNA and RNA degradation (Borkow

and Gabbay 2005). Weaver et al. reported the role Cu metal surface in rapid killing of Methicillin-Resistant Staphylococcus Aureus (antibiotic resistant bacterial infection) (Weaver, Noyce et al. 2010). Their study shows that Cu surface compromises cellular respiration and cause DNA damage (Weaver, Noyce et al. 2010).

Copper pesticides are widely used in agriculture for crop protection (Voulvoulis, Scrimshaw et al. 1999). Aggressive and prolonged use of Cu pesticides produces a high risk of Cu accumulation in fertile soil. Cu from contaminated soil could potentially leach out to nearby water reservoirs (such as nearby lakes and rivers) and negatively impact Cu sensitive aquatic species. Evolution of Cu tolerant bacterial pathogens is not uncommon, in fact, bacterial spot disease in tomato can no longer be fully managed due to the development of Cu resistance. While there is no suitable alternative to Cu available to date for agricultural use, improving Cu efficacy as well as its bioavailability are promising options. Most commercial Cu products (such as Cu oxides, Cu hydroxides, Cu oxychlorides) are water-insoluble and non-phytotoxic. Cu bioavailability in these compounds is therefore limited. Cu bioavailability in water soluble Cu compounds is high, however, they exhibit phytotoxicity. It is highly desirable to develop Cu pesticides, which will have the following physico-chemical properties: (i) superior rainfastness, (ii) non-phytotoxic, (iii) improved Cu bioavailability and (iv) improved antimicrobial efficacy.

In this paper, we present a strategy for improving efficacy through loading of Cu on solid support, this forming a core-shell (C-S) structure, where the silica core serves as an inert and the active Cu is distributed on the shell part. The purpose of designing a core-shell structure with an inert (non-copper) core and copper-silica shell was to reduce copper per spray application without compromising its efficacy. This is based on the fact that Cu pesticides are film-forming material. Bacterial pathogens are inactivated and killed once they come in contact with the film, thus preventing infection.

2. Materials and Methods

2. 1. Materials

All reagents were purchased from commercial vendors and used without any further purification. Ethanol (95% V/V; Fisher Scientific), tetraethylorthosilicate (TEOS; Fisher Scientific), ammonium hydroxide (NH_4OH; 28 – 30 wt% ammonia; Sigma-Aldrich), concentrated hydrochloric acid (Fisher Scientific), copper sulfate pentahydrate (CQ concepts, Ringwood, IL), sodium chloride (Fisher Scientific). Kocide®3000, a product of DuPont ™ was received as a gift from Professor James H. Graham (University of Florida - Citrus Research and Education Center, Lake Alfred, FL, USA). Mueller-Hinton (MH) agar, Luria Bertani (LB) broth and agar for antibacterial study were purchased from Sigma Aldrich. *E. coli* strain ATCC 35218 and *B. subtilis* strain ATCC 9372 were provided by the Microbiology lab, University of Central Florida. Hydrion paper (Fisher Scientific) was used for pH measurements. Nanopure deionized water (Barnstead) was used throughout the study.

2. 2. Methods

Characterization

The characterization of particle size and morphology was done using Zeiss ULTRA-55 FEG Scanning Electron Microscopy. Dynamic Light Scattering (DLS; Precision detector/Coolbatch 40T) technique (Precision detector) was used to estimate particle size (average hydrodynamic diameter) and size distribution of particles in aqueous suspension. Loading of copper was quantified by Atomic Absorption Spectroscopy (AAS; Perkin Elmer Analyst 400 AA flame spectrometer). Turbidity for antibacterial study was measured with Teysche800 spectrophotometer and the intensity of resorufin dye (produced after reduction of resazurin) was measured with a fluorescence spectrometer (Nanolog; HORIBA Jobin Yvon).

Sample preparation for SEM was done by spin coating nanoparticle solution on silicon wafers. Atomic absorption spectroscopy (AAS) analysis was done by comparison with a series of copper standards. Sample preparation involved extraction of Cu from lyophilized copper-loaded silica

nanoparticle powder using saturated ethylenediaminetetraacetic acid (EDTA) solution. The EDTA leaches out Cu from the C-S CuSiNP material, forming water-soluble Cu-EDTA complex

Antibacterial Assays

Bacterial growth inhibition test using turbidity and two standard biochemical assays (Resazurin and Baclight assays) were performed to determine antibacterial properties of C-S CuSiNP material against a gram positive *Bacillus subtilis* (*B.subtilis*, ATCC 9372) and a gram negative *Escherichia coli* (*E.coli*, ATCC 35218) organism. A single colony was inoculated in 10 mL of the broth and grown overnight at 37 ^{o}C on a 150 rpm shaker. Subcultures were periodically made on LB agar plates to maintain the organisms. Kocide® 3000 (Cu hydroxide nanoparticles, represented as "insoluble" Cu compound) and copper sulfate with same metallic copper concentration were used as positive control and silica nanoparticle (SiNP without Cu loading) was used as negative control.

Disk Diffusion Assay

Blank paper disks were saturated in 5 mL of the C-S CuSiNP solution. The disks were dried in vacuum overnight. The dried disks were then placed on Mueller – Hinton agar plated already spread with bacteria. The plates were kept inverted at 37 ^{o}C. After 24 hours, the zone of inhibition was measured. SiNP was used as the negative control.

Bacterial Growth Inhibition – Turbidity Assay

Different concentrations of copper-loaded silica nanoparticles were made in LB broth to a final volume of 10 mL. 10^5 cells/mL of the bacteria were added to all tubes. Silica ("seed") nanoparticle (without Cu loading) was taken as the negative control. Different concentrations of Kocide® 3000 and copper sulfate with same metallic concentration as in copper-loaded silica nanoparticles were considered as the positive control. Since copper-loaded silica nanoparticles, SiNP and Kocide® 3000 are turbid in nature and can interfere with the optical density reading, the background measurements were subtracted to calculate the final reading. All the tubes were shaken well and incubated at 37^{o}C on a 150 rpm shaker. After 24 hours, aliquots were taken to measure the optical density at 600 nm.

Minimum Inhibitory Concentration (MIC) Determination

Resazurin assay method was used to determine the MIC of C-S CuSiNPs against *E. coli and B. subtilis(Sarker, Nahar et al. 2007).* A 12 well cell culture plate was labeled. Different concentrations of C-S CuSiNP (2.45 to 14.7 ppm copper concentartion) were added to the wells. Silica nanoparticle was taken as the negative control. The total volume in all wells was made to 3mL with autoclaved water. 10^5 cells /mL of bacteria was added to all the wells. 150 µL of resazurin dye solution (6.75mg/mL) was then added to the wells. One well was maintained as control for resazurin (without bacteria and sample) and another well as control for the bacteria (without sample but with resazurin). The plate was sealed with parafilm and incubated on a 150 rpm shaker at 37 ^{o}C. Since the C-S CuSiNP was turbid in nature, intermediate colors were obtained. So the fluorescence property of resofurin was taken into account and the intensity of fluorescence was measured to find the MIC.

2. 3. Experimental

Synthesis of silica nanoparticles

Silica nanoparticles (SiNP) were synthesized (following Stöber sol-gel method with further modification (Thomassen, Aerts et al. 2009)) by addition of the reagents in the same order (1 to 4) as shown in **Table 1** and stirred on the magnetic stirrer for 24 hours at 400 rpm. After 24 hours, the nanoparticle solution was isolated by centrifugation at 10,000 rpm for 10 minutes and purified by washing three times with de-ionized (DI) water. The final pellet was dispersed in 100 mL DI water and stored at room temperature.

Synthesis of copper –loaded silica nanoparticles (CuSiNP)

The copper – loaded silica nanoparticles (CuSiNP) were synthesized by adding the reagents in the order 1 to 4 as mentioned in **Table 1** (Thomassen, Aerts et al. 2009). Then 593.35 mg of copper sulfate was added. The reaction mixture was then allowed to stir on a magnetic stirrer at 400 rpm. After 24 hours, the particles were thoroughly washed (5x) with water. Centrifugation (10,000 rpm for 10 minutes) and

vortex techniques were used between two washing steps. The CuSiNPs were then re-suspended in DI water for further use.

Synthesis of copper –loaded silica nanoparticles (C-S CuSiNP)

The CuSiNP synthesis procedure was further modified by the addition of TSPETE (N-(Trimethoxy Silyl Propyl) – Ethylenediamine Triacetic acid, Trisodium salt 45% in water). 1.25 mL TSPETE (Rastogi, Rutledge et al. 2011) was added to the synthesized copper-loaded silica nanoparticles and stirred magnetically at 400 rpm for 4 hours. The solution separated into two parts – light blue solution (top portion) and a blue precipitate (bottom part). The top portion was discarded. The bottom part was collected, thoroughly washed (5x) with DI water and then re-dispersed in DI water. Centrifugation and vortex techniques were used between two washing steps.

Table. 1. Synthesis of silica nanoparticles (SiNPs).

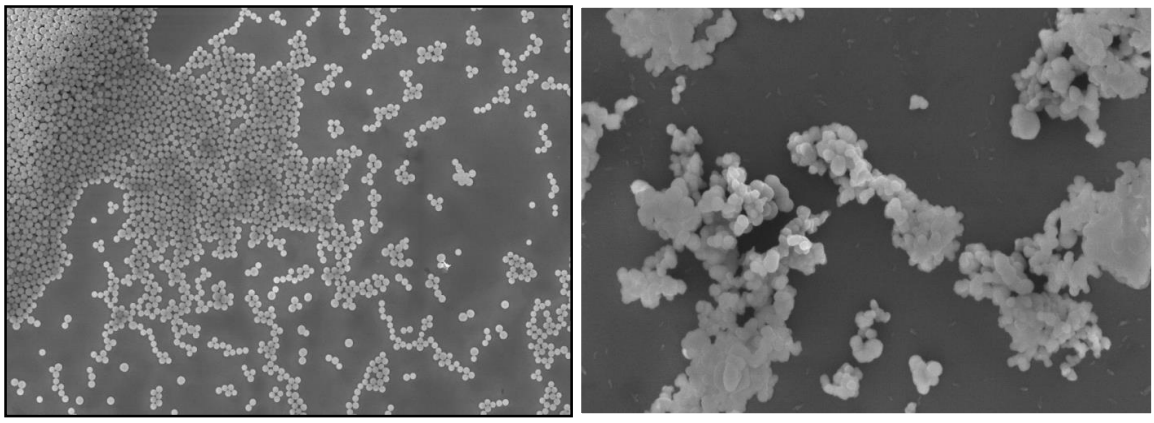
Serial #	Reagent	Volume added (mL)
1	Ethanol	100
2	Water	2.97
3	Tetraethylorthosilicate	3.78
4	Ammonium hydroxide	4.85

3. Results and Discussion

CuSiNP

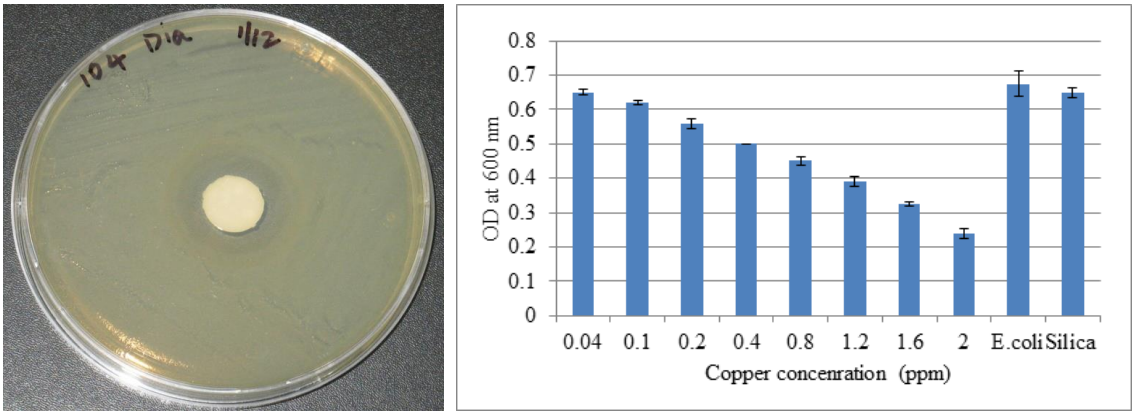
SEM image revealed the formation of highly monodispersed spherical SiNPs with smooth surface morphology and the average particle size was estimated to be 107 nm for SiNP (**Figure 1a**). These particles were well dispersed in DI water due to negative surface charge (deprotonated silanol groups). SEM image showed aggregated particles and the average particle size was estimated to be 200 nm for CuSiNPs **(Figure 1b)**. This is due to binding of positively charged Cu(II) ions to SiNP surface (complexed with Si-OH and $Si\text{-}O^-$ groups), reducing the overall surface charge. The amount of copper was estimated to be 0.4 ppm from atomic absorption spectroscopy (AAS).

Disk diffusion assay resulted in a zone of inhibition of ~18mm **(Figure 2)** showing that copper diffused out of the nanoparticle matrix and inhibited growth of *E. coli*. Inhibition of growth at different concentrations of CuSiNP (0.04 to 2 ppm) was evaluated after 24 hours of incubation at 37ºC by measuring the optical density at 600nm. Significant inhibition of growth of *E. coli* was observed in the presence of CuSiNP when compared to SiNP (no copper) **(Figure 3)**. However, total inhibition could not be achieved due to less copper retention in CuSiNP.



(a) (b)

Fig. 1. SEM image of (a) SiNP and (b) CuSiNP

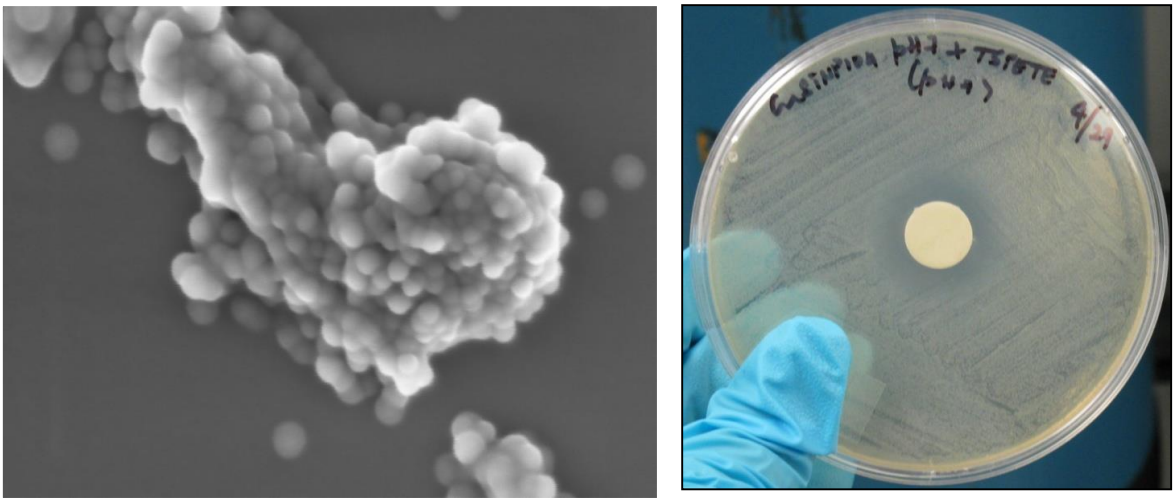


(a) (b)

Fig. 2. (a) DDA plate showing zone of inhibition for copper-loaded hybrid silica nanoparticles and (b) Histogram for inhibition of *E. coli* by CuSiNP.

C-S CuSiNP

Copper retention in C-S CuSiNP was not significant. C-S CuSiNPs were formed as aggregates and the dispersibility was very low. To overcome these limitations, TSPETE, a carboxyl based surface modifier and copper chelator was added to pre-synthesized C-S CuSiNP (Yang, Santra et al. 2006). Few minutes after the addition of TSPETE, blue gel-like substances began to separate from the solution and stick to the sides and bottom of the container. The top solution was light-blue in color and the bottom gel-like substance was deep blue in color and highly water soluble. DLS analysis of both the solutions revealed the top solution had particle size in microns and the bottom-solution had particles of ~ 105 nm in size. The bottom gel-like substance was considered for further study.



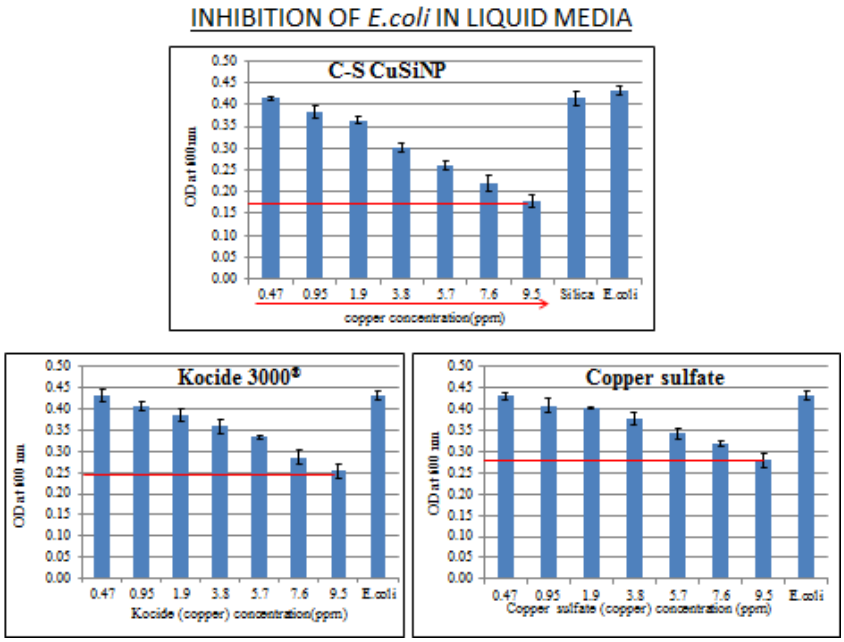
(a) (b)

Fig. 3. (a) SEM image of C-S CuSiNP and (b) DDA plate showing zone of inhibition for C-S CuSiNP.

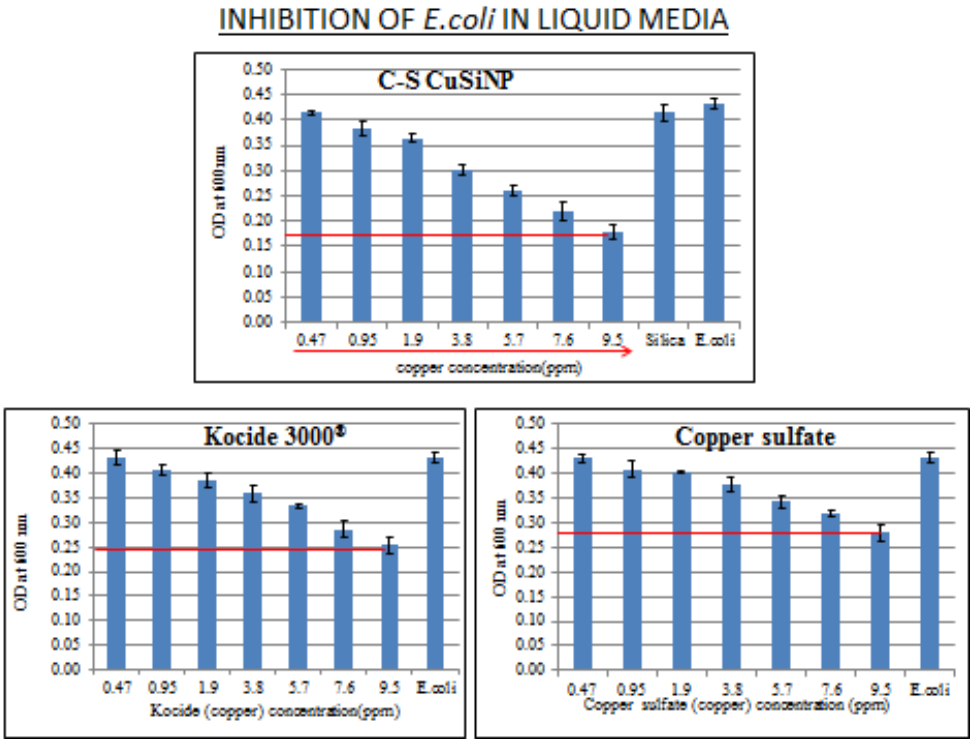
DLS measurements estimated the average particle size of C-S CuSiNP to be ~ 105 nm. SEM image showed aggregates of C-S CuSiNP with individual particle size of ~50 nm **(Figure 3a)** whereas the aggregates are in submicron range. Based on AAS, the amount of metallic copper was estimated to be 1.9 ppm.

Disk diffusion assay gave a zone of inhibition of ~ 20 mm showing the diffusion of Cu from the silica matrix **(Figure 3b)**. Inhibition of growth of *E. coli* and *B. subtilis* was performed in LB broth by measuring turbidity at 600 nm after a 24 hour incubation period at 37 ^{o}C. As the concentration of copper

increased, the growth of both *E. coli* and *B. subtilis* decreased **(Figure 4)** when compared to the silica nanoparticle (without copper), negative control. Total inhibition was not achieved due to reduced copper loaded in the C-S CuSiNP.



(a)



(b)

Fig. 4. Histogram showing inhibition of *E. coli* (a) and *B. subtilis* (b) by the C-S CuSiNP, Kocide 3000 and Copper Sulfate.

Plant tissue injury potential (phytotoxicity) study of CS-CuSiNPs at pH 7.0 was carried out in a Panasonic Environmental Test Chamber (Model MLR- 352H) which allowed for controlled day/night cycling temperatures, light intensity and humidity to simulate summer weather conditions (biocide application season with average high temperature of 85 degrees Fahrenheit and relative humidity 60-80%). Studies conducted on *Vinca sp*, an ornamental plant revealed no sign of plant injury even as high as 900 ppm of metallic Cu. Copper sulfate was used a positive control known to cause plant injury while Kocide 3000 acted as a negative control known to cause no damage. Table 2 summarizes phytotoxicity results.

Table. 2. Phytotoxicity evaluation of C-S CuSiNP.
("-" non-phytotoxic; "+" moderately phytotoxic; "++" severely phytotoxic)

	Metallic Cu (ppm)	**Time (hr)**		
		24	48	72
CS-CuSiNP	900	-	-	-
$CuSO_4$	900	+	++	++
Kocide® 3000	900	-	-	-
SiNP	-	-	-	-

4. Conclusion

Copper-loaded silica nanoparticles were synthesized in two different approaches. In the first approach, CuSiNP was synthesized in a single step. Cu loading was not significant (0.4 ppm). To improve Cu loading, the surface of CuSiNP was further modified with water soluble TSPETE reagent, thus forming C-S CuSiNP. Cu loading was improved to 1.9 ppm. Overall particle dispersibility of C-S CuSiNP was increased due to surface coating with TSPETE. Antimicrobial efficacy of the C-S CuSiNP was comparable to Kocide 3000 commercial Cu product when evaluated against *E. coli* and *B. subtilis*. The C-S CuSiNP did not show any sign of phytotoxicity.

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