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Antimicrobial Assessment of Cellulose-Copper-Silica Nanocomposites for Crop Disease Management

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***Abstract*—Copper biocides have been widely used in agriculture for centuries, causing plant pathogens to develop resistance and other environmental problems. Previously, copper-silica nanoparticles (Cu-Si NPs) have demonstrated enhanced antimicrobial properties compared to standard copper products. Cellulose is the most abundant biopolymer, it possesses biodegradability, and many of its nanomaterials exhibit antimicrobial properties, leading to the study of novel cellulose-Cu-Si nanocomposites to improve the efficacy of Cu-Si NPs. These materials were assessed against Xanthomonas alfalfae (copper-sensitive pathogen) and Xanthomonas perforans GEV-485 (copper-tolerant pathogen). Results suggest that the composite has potential to minimize copper use in agriculture industry.**

***Keywords*—agriculture, plant disease, copper, silica, cellulose nanocrystals, nanocomposite, crop protection**

I. Introduction

Up to 16% of global crop yield is lost due to plant pathogens [1]. This issue leads to numerous consequences, including lower quality produce and increased food prices; $220 billion is spent solely on combating them each year in the United States alone [2]. With copper being one of the most prominent agents in bactericides for centuries, pathogens have been developing resistance. Recent innovations within nanobiotechnology have formulated copper-derived nanoparticles that exhibit improvements in their antimicrobial ability compared to common copper bactericides [3].

Previously, copper (II) oxide nanoparticles (CuO NPs) have been studied in their effects against Fusarium oxysporum infected tomato plants and Verticillium dahliae infected eggplants [4]. The study concluded that treating plants with CuO NPs correlated with greater copper retention within the plants, increased growth, and a reduction in disease severity over untreated, infected plants. Similarly, copper (II) sulfide nanoparticles (CuS NPs) have displayed promising properties within numerous contexts, including infection control in rice, copper retention in roots, and food packaging additives. A previous study on rice determined a complete fungal growth inhibition at a copper concentration of 15 mg/L against Alternaria alternata, Drechslera oryzae, and Curvularia lunata [5]. Furthermore, a higher level of plant biomass and chlorophyll content was achieved by the CuS NPs than by the CuS control [6]. Copper-zinc oxide nanoparticles (Cu-ZnO NPs) have demonstrated the synergistic ability of multiple antimicrobial metals. Against the plant pathogens Xanthomonas alfalfae and Pseudomonas syringae, Cu-ZnO have demonstrated an enhanced antimicrobial characteristic than those of numerous controls ($CuSO_4$, CuO, ZnO) [7]. A study determined that copper-chitosan nanoparticles significantly reduced Curvularia leaf spot disease in tomato plants [8]. Previous works have developed copper-silica nanoparticles (Cu-Si NPs) that embody silica shell cores coated with copper [9]. Cu-Si NPs demonstrated improved antimicrobial qualities against Escherichia coli, Bacillus subtilis, and Xanthomonas alfalfae at lower concentrations than various materials, including $CuSO_4$ bulk and Kocide® 3000 (a widely used copper-based bactericide) [10]. Furthermore, the study conducted in vitro antimicrobial assays that displayed great ability for Cu-Si NPs to combat both copper-sensitive and copper-tolerant bacterial strains. A previous study indicated antimicrobial activity against X. perforans (a copper-tolerant strain) from Cu-Si NPs, whereas Kocide® 3000 demonstrated limited biocidal activity [11].

Cellulose was utilized in an effort to improve the efficacy of Cu-Si NPs with sustainable resources. Cellulose is readily available, biodegradable, and has been utilized for antimicrobial nanocomposites such as films, and particles, among others [12]–[15]. Moreover, cellulose nanocrystals have been utilized as a crop management tool, showing antimicrobial activity, biodegradability and many other promising properties, making them favorable in environmental-friendly technologies for sustainable agriculture [16]. Their combination with copper nanoparticles poses a novel, antimicrobial material that may better treat plant diseases.

Herein, the proposed cellulose-Cu-Si nanocomposites

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were assessed against X. alfalfae (copper-sensitive plant pathogen) and X. perforans GEV-485 (copper-tolerant plant pathogen). The microbroth dilution method was utilized to assess the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The antimicrobial studies compare a cellulose-Cu-Si nanocomposite (Gamma) to a cellulose-Cu-Si composite (Beta), Cu-Si NPs without cellulose (Alpha), and ionic Cu^{2+}. It was hypothesized that Gamma would display enhanced antimicrobial abilities against both pathogens compared to Alpha, Beta, and Cu^{2+} ion due to the incorporation of cellulose nanocrystals.

II. Materials and Methods

A. Materials

All of the reagents used in this study were purchased from commercial sources and used without further purification: copper (II) hydroxide ($Cu(OH)_2$), 30% ammonium hydroxide (NH_4OH), sodium gluconate ($C_6H_{11}NaO_7$), pure cellulose ($C_6H_{10}O_5$), potassium silicate (K_2O_3Si), 64% sulfuric acid (H_2SO_4), urea (CH_4N_2O), copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), citric acid ($C_6H_8O_7$), nutrient broth (NB), NB agar, Xanthomonas alfalfae bacteria, Xanthomonas perforans GEV-485 bacteria, and deionized (DI) water.

B. Cuoxam Synthesis

Cuoxam was used as the copper source for Beta and Gamma. In brief, 153.0 mg of copper (II) hydroxide was massed into a vial and mixed with 1.9 mL of concentrated ammonium hydroxide, 0.5 mL of DI water, and 93.4 mg of sodium gluconate. The mixture was set to stir for 1 hour before being used in nanocomposite synthesis.

C. Alpha Synthesis

To synthesize Alpha (Cu-Si NPs), 73 mL of 30% ammonium hydroxide, 22 g of urea, 4.3 mL of DI water, and 1.62 g of potassium silicate were added to a flask. The content was then left to stir for two hours at 70°C. This was used to formulate spherical silica cores. After the solution cools down, 5.9 g of copper sulfate and 136.0 mg of citric acid was dissolved in 7.5 mL of DI water. This solution was then combined to the spherical silica cores, forming the Cu-Si NPs. The volume was then adjusted to have a final concentration of 20,000 ppm Cu.

D. Beta Synthesis

To synthesize the Cu-Si composite, 50.0 mg of pure cellulose is reacted with Cuoxam at room temperature, until dissolution. Afterwards, 35.0 mg of potassium silicate is added to the flask and left to stir for three hours. The sample is then adjusted to a pH of 10 and to 20,000 ppm Cu.

E. Gamma Synthesis

To synthesize Gamma, cellulose nanocrystals were first created through acid hydrolysis. In brief, 1.50 g of cellulose were treated with 15 mL of 64% sulfuric acid and set to stir for 40 minutes. The solution was then centrifuged at 12,000 rpm and resuspended in 15 mL of DI water. An aliquot of 0.5 mL of the cellulose nanocrystals suspension was added to Cuoxam. After mixing, 35.0 mg of potassium silicate was added to the suspension. The sample was then left to stir for three hours. Lastly, the sample was adjusted to a pH of 10 and to 20,000 ppm Cu.

F. Pathogen Incubation

The bacteria were first obtained from frozen stocks stored at -80°C and then plated onto a NB agar plate using inoculation loop. The plate was then sealed with parafilm and placed in an incubator at 28°C for 72 hours to grow. Afterwards, an inoculation loop was used to capture several independent colonies from the plates and inoculate a flask containing 10 mL of nutrient broth. The flask was then placed into a stirring incubator at 28°C for 48 hours.

G. Minimum Inhibitory Concentration (MIC) Assessment

The MIC was determined by utilizing a 96 well plate and the microbroth dilution method. In brief, a serial dilution with NB was performed for each treatment (512 to 0.5 ppm Cu) by sequentially transferring half the volume of the wells. A nanoparticle stock solution with a concentration four times greater than the highest concentration being tested was utilized, given that the addition of broth and bacteria would lead to a four-fold dilution in each well. Therefore, a stock solution of 2,048 ppm Cu was used for the serial dilutions.

Separately, the bacterial broth was standardized by measuring the optical density at 600 nm (OD600) using a spectrophotometer and diluting with NB until approximately 10^8 colony forming units per mL (0.1 for X. alfalfae; 0.3 for X. perforans). Afterwards, a 100-fold dilution of the standardized OD bacteria was conducted and the well plate was inoculated. Afterwards, the plate was secured using parafilm and placed in a pathogen incubator at 28°C for 40-48 hours. Bacterial growth was assessed by measuring the OD600 in a microplate reader. The MIC was determined by a 70% reduction in the OD600 compared to the growth control. All treatments were evaluated in triplicates and the experiments were each reproduced three times (n=9).

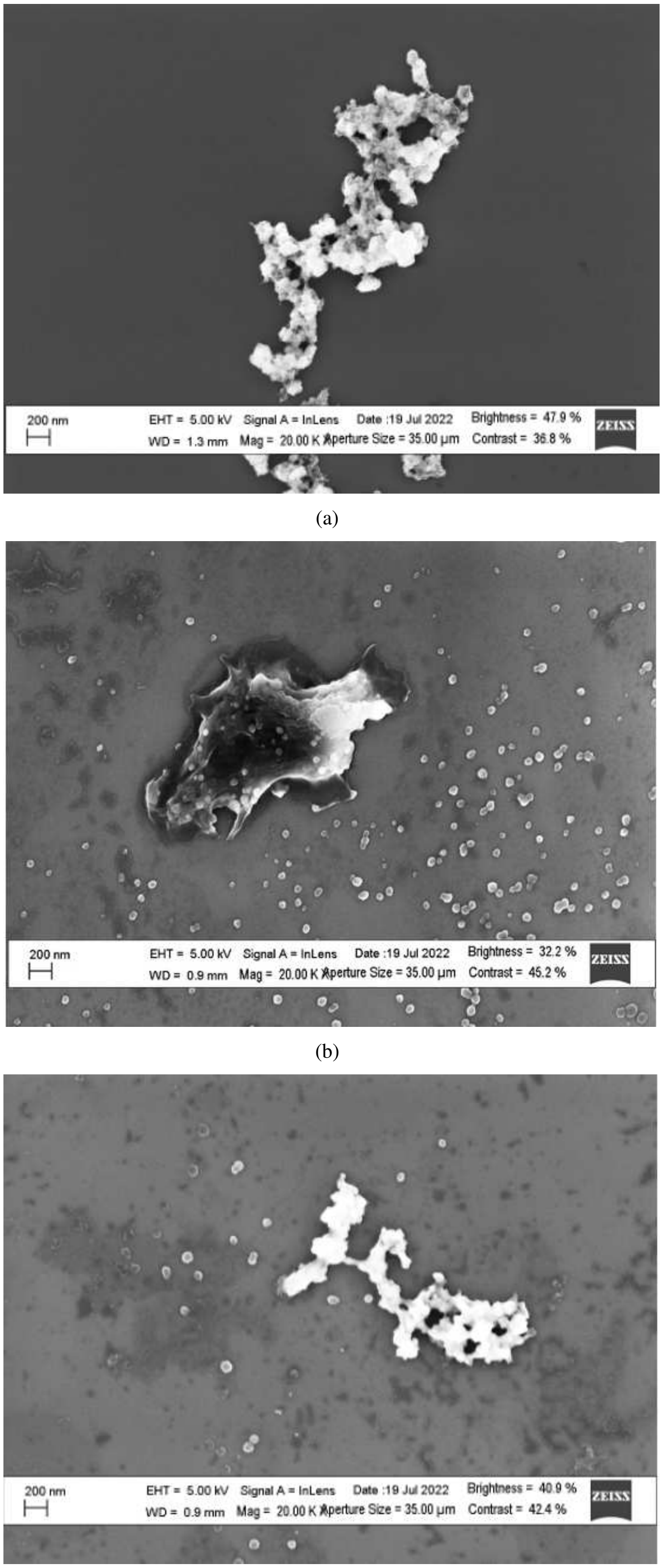
H. Minimum Bactericidal Concentration (MBC) Assessment

The minimum bactericidal concentration (MBC) assay was conducted from the same well plate utilized in the MIC assessment. Starting from the MIC, the solutions in the wells were plated on NB agar plates and incubated for 40-48 hours at 28°C. The absence of bacteria colonies on the agar plates suggest bacterial death at the specified concentration, which was used to determine the MBC.

III. Results and Discussion

A. Copper Nanoparticles Characterization

The scanning electron microscope (SEM) images suggested various characteristics regarding shape, size, and possible function of the different copper nanoparticle samples.



(a) (b) (c)

Fig. 1. At 20 ppm Cu and 20.00K magnification, SEM images of (a) Alpha, (b) Beta, and (c) Gamma.

Alpha's SEM image (Fig. 1a) indicated porous submicron-sized agglomerates of Cu-Si NPs, possessing sizes of less than 200 nm. Beta shows individual Cu-Si NPs on micro-sized cellulose agglomerates (Fig. 1b), complying with the Cu-Si cellulose NPs development that is to be expected. Fig. 1c displays Cu-Si NPs surrounding cellulose nanocrystal agglomerates. It is anticipated that the sizes and structures of all three samples would enable them to stay in a plant after application. This would lead to a buildup of copper within the plant, which could catalyze plant processes (eg. photosynthesis and cellular respiration) to stimulate growth. That being said, copper toxicity levels within the plant must also not be reached. The micron-sized structures present in Alpha, Beta, and Gamma may also assist in the prevention of copper runoffs and copper contamination of the soil/environment, as opposed to Cu^{2+} solutions that can more easily wash through plants in the presence of external factors (eg. rain).

B. MIC Results and Interpretation

Measured at a wavelength of 600 nm, the optical density (OD) is used to indicate the concentration of bacteria present, where a higher OD signifies more bacteria. The MIC results (Fig. 2) indicate a lowering of OD as concentrations first increase, then a sudden increase in OD at high copper concentrations (64 ppm Cu to 128 ppm Cu). This rise in OD is due to observed nanoparticle precipitation.

The MIC of Alpha, Beta, Gamma, and Cu^{2+} are 16 ppm Cu, 16 ppm Cu, 16 ppm Cu, and 32 ppm Cu, respectively (Table 1). Against X. perforans, the MIC of Alpha, Beta, Gamma, and Cu^{2+} are 64 ppm Cu, 16 ppm Cu, 16 ppm Cu, and 128 ppm Cu, respectively (Table 1). Alpha, Beta, and Gamma displayed an improved MIC compared to Cu^{2+}. Furthermore, against X. perforans GEV-485, Beta and Gamma displayed no change in MIC, suggesting high efficiency in growth inhibition capabilities. Alpha displayed a much higher MIC, suggesting the incorporation of cellulose improved the antimicrobial characterization of Cu-Si NPs.

C. MBC Results and Interpretation

The MBC is determined by plating the solutions from the 96-well plate onto NB agar to look for bacteria growth. The lowest concentration with no bacteria growth is the MBC. For X. alfalfae, the MBC of Alpha, Beta, Gamma, and Cu^{2+} are 16 ppm, 32 ppm, 32 ppm, and 64 ppm, respectively (Table 2). This suggests up to a four-fold improvement from Cu^{2+} solution by the copper nanoparticles. The MBC results for X. alfalfae are contrary to expectations, since they suggest the Cu-Si NP performs better in killing the bacteria strain than the cellulose-Cu-Si composite and the cellulose-Cu-Si nanocomposite. Against the copper-tolerant strain X. perforans, the MBC of Alpha, Beta, Gamma, and Cu^{2+} are 128 ppm, 64 ppm, 32 ppm, and 256 ppm, respectively. Alpha, Beta, and Gamma still maintained a lower MBC than that of Cu^{2+}, which better supports the claim that these copper nanoparticles perform better in an antimicrobial sense than Cu^{2+} solution. Furthermore, Gamma having the lowest MBC

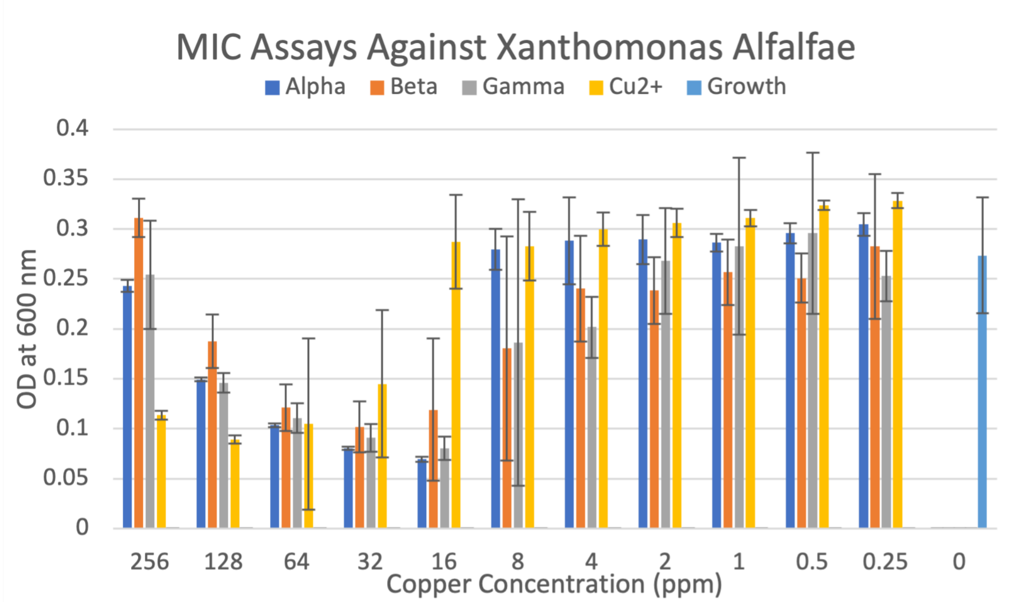


Fig. 2. MIC assay results from 3 trials of Alpha, Beta, Gamma, and Cu^{2+} against Xanthomonas alfalfae at different copper concentrations (0 ppm Cu to 256 ppm Cu)

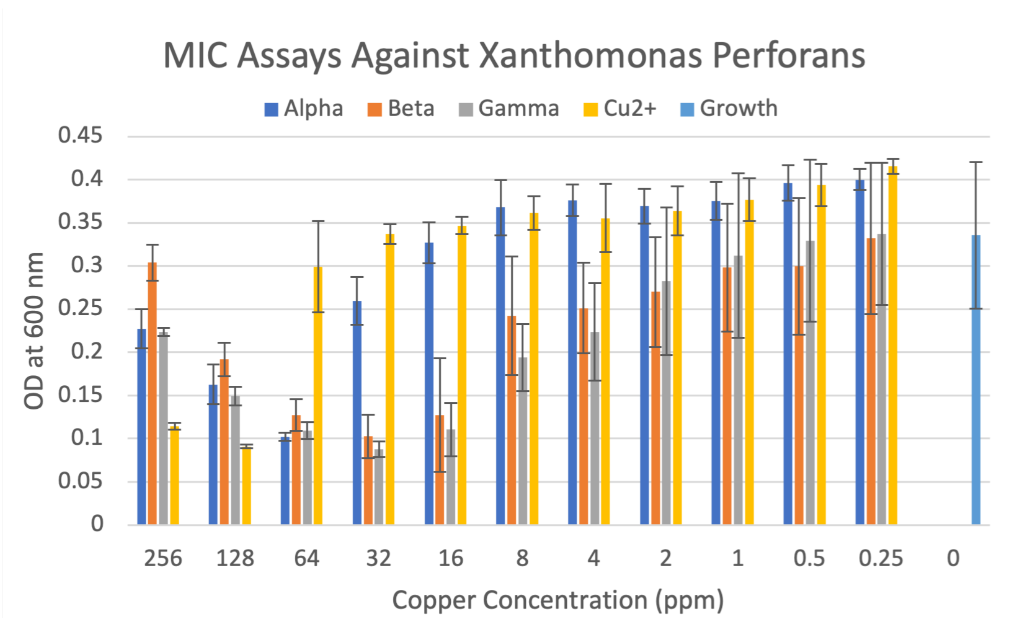


Fig. 3. MIC assay results from 3 trials of Alpha, Beta, Gamma, and Cu^{2+} against Xanthomonas perforans at different copper concentrations (0 ppm Cu to 256 ppm Cu)

TABLE I
MIC RESULTS OF ALPHA, BETA, GAMMA, AND Cu^{2+} AGAINST X. ALFALFAE AND X. PERFORANS

Treatment Bacteria	Alpha (ppm Cu)	Beta (ppm Cu)	Gamma (ppm Cu)	Cu 2+ (ppm Cu)
X. alfalfae	16	16	16	32
X. perforans	64	16	16	128

TABLE II
MBC RESULTS OF ALPHA, BETA, GAMMA, AND Cu^{2+} AGAINST X. ALFALFAE AND X. PERFORANS

Treatment Bacteria	Alpha (ppm Cu)	Beta (ppm Cu)	Gamma (ppm Cu)	Cu 2+ (ppm Cu)
X. alfalfae	16	32	32	64
X. perforans	128	64	32	256

(32 ppm Cu) indicates the antimicrobial benefit of having cellulose nanocrystals against X. perforans. Beta having the second lowest MBC (64 ppm Cu) suggests the overall advantage of including cellulose among the Cu-Si NPs in killing X. perforans. Although Alpha had the lowest MBC against X. alfalfae, it had a much higher MBC with X. perforans. This may indicate a drawback of Cu-Si NPs in real world applications, as their performance against a copper-tolerant pathogen is incredibly inefficient compared to their performance against a copper-sensitive pathogen (MBC of 128 ppm Cu against X. perforans versus MBC of 16 ppm Cu against X. alfalfae).

IV. CONCLUSION

The Cu-Si cellulose NPs (Beta) and Cu-Si cellulose nanocrystal NPs (Gamma) demonstrated significantly improved inhibitory and bactericidal properties over the Cu^{2+} solution. Compared to Cu-Si NPs alone (Alpha), Beta and Gamma demonstrated much greater antimicrobial characterization against the copper-tolerant X. perforans strain, despite Alpha having the lowest MBC against the copper-sensitive X. alfalfae strain. The size and shape characterization of Beta and Gamma indicate possible future studies in determining plant disease target specialization. With a promising antimicrobial assessment against X. perforans, Gamma suggests the possibility of a novel, applicable copper nanoparticle within the agricultural field to serve as an effective alternative to today's common bactericides. Further studies are needed to determine the bactericidal mode of action for the cellulose-Cu-Si nanocomposites against copper-tolerant bacteria.

V. CONFLICTS OF INTEREST

The author(s) declare no conflicts of interest with respect to any element of this article, including the research, authorship, and publication.

VI. ACKNOWLEDGEMENTS

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