Engineered core-shell Cu particles demonstrate strong potential for plant disease control

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ABSTRACT

Copper (Cu) bactericides/fungicides are used extensively for crop protection in agriculture, leading to accumulation and antimicrobial resistance. Increasing Cu bioavailability can reduce the amount required for crop protection. We have developed Core-Shell-Cu particles (CS-CuPs) containing an inert silica core coated with a Cu silica shell. Electron microscopy results confirmed the formation of spherical sub-micron size particles. Field efficacy of CS-CuPs was evaluated by assessing its ability to protect citrus trees from canker infection. Analysis of 2014 citrus canker infection on "Ray-Ruby" grapefruit showed a 62.8% incidence of fruit lesions on untreated trees while commercial copper hydroxide reduced incidence to 16.4% at a rate of 1.0 kg metallic Cu/hectare. The CS-CuPs reduced canker incidence to 15.6 % at 0.22 kg metallic Cu/hectare. Maintenance of efficacy at a significantly lower metallic Cu rate (about 4.5 times) clearly demonstrates the potential of CS-CuPs for successful use as an agricultural bactericide/fungicide.

Keywords: Core-shell, Copper, Silica particle, Antimicrobial, Silica gel, citrus canker

1 INTRODUCTION

Copper bactericides/fungicides are the most commonly used metal-based crop protectants for managing bacterial and fungal diseases of a wide variety of crops including citrus, vegetables, stone fruit, legumes cereals, pome fruit and berries [1-3]. Application rates and frequency vary per crop as well as climate conditions. For instance, citrus crop receives approximately 5-15 applications per season, starting from March through October [4-5]. Long-term use of Cu has several limitations: (i) Soil Cu level increase that reduces uptake of other essential elements such as Zn, (ii) development of Cu resistance to target microbial species and (iii) higher chance of Cu toxicity to beneficial soil microbiome.

Most commercial Cu products such as Cu oxides, Cu hydroxides, Cu oxychlorides are sparingly soluble in water and therefore form a good film after foliar application. Low solubility greatly minimizes the risk of phytotoxicity but also reduces Cu bioavailability. In contrast, soluble form of Cu such as Cu chelates, Cu salts have higher bioavailability but causes severe phytotoxicity if applied at the rate of film-forming Cu. To address these limitations of Cu phytotoxicity and bioavailability, we have developed Cu loaded silica particles with a core-shell structure, CS-CuPs. In the CS-CuP system, a shell of Cu loaded silica nanogel coats silica core that serves as an inert. C-S CuP serves as a combination of both soluble and insoluble Cu compounds where the silica matrix serves as a Cu delivery system.

We hypothesize that Cu embedded only in the shell region of the C-S CuP will reduce the amount of Cu per application without compromising antimicrobial efficacy. This present particle design is effective in maintaining efficacy but reduces Cu release into the environment.

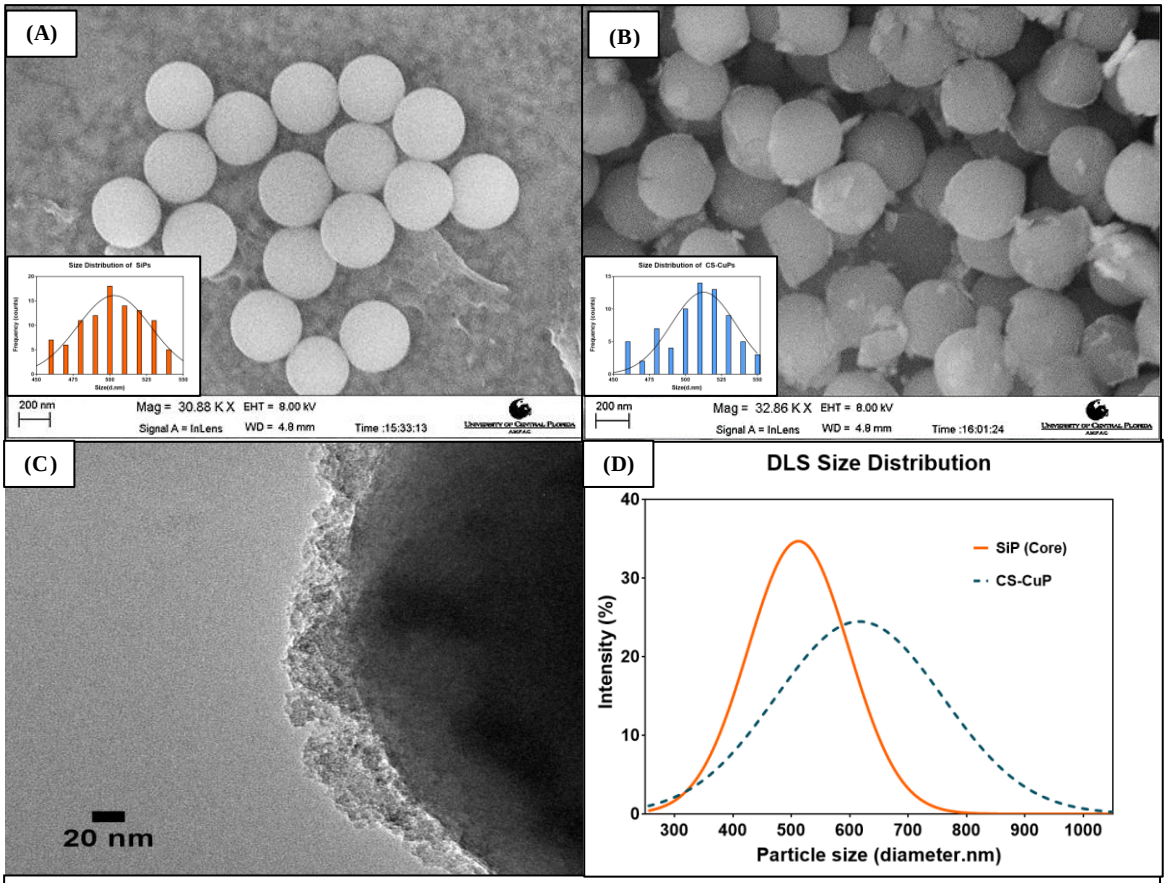

Figure 1: (A) SEM image of silica particles (SiPs). These particles are in the sub-micron size range, 350-600 nm and spherical in shape. Inset displays size distribution of SiPs (individual particle size was measured using ImageJ software; more than one SEM image was used). (B) SEM of CS-CuPs showed deposition of gel particles on CuP, forming particles with semi-spherical shape with particle size range, 350 - 600 nm. Inset displays size distribution of CS-CuPs. (C) High magnification HRTEM image of an individual CS-CuP reveals the outer shell of Cu-silica over the inert silica core (from difference in contrast), with varying shell thickness between ~20-40nm. (D) Hydrodynamic diameter of the particles size distribution of SiP core and CS-CuPs as measured by the DLS. The overall particle size and size distribution are increased after coating the silica core with a copper-silica shell.

2 METHODOLOGY

2.1 Synthesis of Core-Shell Cu particles

Core-shell Cu particles were synthesized following our previously published protocol with some modifications [6]. In our earlier work, we confirmed that creating shell of Cu and silica over an inert silica core increased Cu bioavailability and its overall *in vitro* antimicrobial effectiveness. The silica core particle was synthesized using a Stober synthesis method [7]. In a typical procedure, tetraethyl orthosilicate (TEOS, a silane reagent) is hydrolyzed using ammonium hydroxide base in ethanol-water. In our previous method, following a seeded growth process, silica core particles were purified prior to growing a Cu-loaded shell layer over the core particles. This process is cumbersome and not a practical process for handling large scale synthesis to support field trial. Therefore, in the present method, the Cu-loaded silica gel was synthesized separately using acid-hydrolyzed sol-gel process using TEOS, copper salt and hydrochloric acid. In the next step, under mechanical stirring conditions, the solution containing silica core particles and the solution containing Cu-loaded silica gel particles were mixed together. Stirring was continued overnight for allowing growth of Cu-silica gel coating over the silica core particles. The pH of the mixture was close to neutral and the concentration of metallic Cu was 25,450 µg/mL.

Material	MIC (µg/mL metallic Cu)			
	Xanthomonas alfalfae subsp. *citrumelonis* (ATCC 49120)	*Pseudomonas syringae* pv. *syringae* (ATCC 19310)	*Clavibacter michiganensis* subsp. *michiganensis* (ATCC 10202)	*Escherichia coli* (ATCC 10536)
CS-CuPs	125-250	125-250	125	125-250
Copper hydroxide	250-500	250	125-250	500
Copper sulfate	125-250	250	125-250	250-500

Table 1: MIC values of CS-CuPs demonstrate the in-vitro bactericidal activity compared with copper hydroxide and copper sulfate standards for disease control

2.2 Characterization of CS-CuPs

The particle size, dispersion and morphology of CS-CuPs were determined using Scanning Electron Microscopy (SEM), High Resolution Transmission Electron Microscopy (HRTEM) and Dynamic Light Scattering (DLS). Samples were prepared for both SEM and HRTEM by vortexing and drop casting onto a carbon coated gold TEM grids and allowing to air-dry overnight under sterile conditions inside a laminar flow hood. SEM analysis was conducted on a Zeiss ULTRA-55 FEG SEM while HRTEM was conducted on a FEI Tecnai F30 TEM. DLS was conducted on a Malvern Zetasizer Nano ZS. DLS samples were vortexed and sonicated for 5 minutes before scanning the sample with the Nano ZS. Multiple dilutions were analyzed to get a clear understanding of the hydrodynamic size.

2.3 Antimicrobial Evaluation

The antimicrobial properties of the CS-CuPs was studied by determining the Minimum Inhibitory Concentration (MIC). Samples were tested against Gram negative *Xanthomonas alfalfae* subsp. *citrumelonis* strain F1 (ATCC 49120, a citrus canker surrogate), Gram negative *Pseudomonas syringae* pv. *syringae* (ATCC 19310, causative agent of bacterial speck in Lilac, almond, apricots, peaches and wild beans among others) and Gram positive *Clavibacter michiganensis* subsp. *michiganensis* (ATCC 10202, causative agent of bacterial wilt and canker in *Tomato sp*). *X. alfalfae* and *P. syringae* were maintained with nutrient agar and broth while *C. michiganensis* was grown with brain heart infusion (BHI) media. All bacteria were grown at 26 ºC. CS-CuPs was compared to copper hydroxide and copper sulfate.

The MIC values of CS-CuPs along with copper hydroxide and copper sulfate were carried out using broth microdilution in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [8]. The accuracy of the MIC determination was improved by adding 10 µL of resazurin dye (0.0125 % w/v) per 100 µL well volume and observing color changes (blue to pink for live organisms). This reduced the error that may result from only observing turbidity for bacterial growth because of the turbidity also produced by copper hydroxide and CS-CuPs.

2.4 Citrus Canker Field Trial

In 2014, a field trial was conducted with 7-year-old non-bearing 'Ray Ruby' grapefruit trees in Ft. Pierce, St. Lucie County, FL. The tree spacing was 12 ft x 25ft (145 trees per acre). The experimental design was a randomized complete block design with multiple treatments replicated 5 times in blocks of five contiguous trees. The untreated check (UTC) trees received a water-only spray treatment at each foliar spray time. Materials were mixed with water and applied as foliar sprays at 3.0 L per tree with a handgun sprayer at 1380 kPa of air pressure. Treatments were initiated after the spring flush in April 2014. Materials were sprayed at roughly 21 day intervals on 4/1, 4/22, 5/12, 6/2, 6/23, 7/14, 8/4, 8/25, 9/15, 10/6. Disease evaluation was on 10/20/14. Disease incidence: the incidence of fruit with canker lesions was assessed for 100 fruit per treatment from the middle 3 trees in each plot. Lesions were classified as "old" if they were larger than 0.25 inches in diameter, coalescing with surrounding lesions, black in color, exuding gum or had a prominent yellow halo; and "young" if lesions were smaller than 0.25 inches in diameter, brown in color, and were not coalescing with surrounding lesions. Disease severity was rated for each fruit based on the estimated number of old or young lesions: 1= 0 lesions, 2= 1-5 lesions, 3= 6-20 lesions, 4= 21 or more lesions. Monthly rainfall in 2014 was recorded at University of Florida/ IFAS, Indian River Research and Education Center, Ft. Pierce, FL site, and obtained from the Florida Automated Weather Network website (http://fawn.ifas.ufl.edu/). The monthly rainfall was compared to the average for the last 10 years.

Treatment	Metallic Cu (kg/ha)	Incidence old lesions (%)	Incidence young lesions (%)	Total incidence (%)
Untreated check (UTC)	-	45.0 a	17.8 a	62.8 a
Copper hydroxide	1.0	11.8 bc	4.6 b	16.4 cde
Cuprous oxide	1.12	16.8 b	4.4 b	21.2 bcd
Cuprous oxide	2.24	10.2 bc	5.8 b	16.0 cde
Copper sulfate	0.16	12.4 bc	6.0 b	18.4 bcd
CS-CuPs	0.22	11.6 bc	4.0 b	15.6 cde

[z] Treatments followed by unlike letters are significantly different at P ≤ 0.05 according to Student-Newman-Keuls multiple range test

Table 2: Effect of CS-CuPs on incidence of citrus canker-infected fruit with old lesions, young lesions and total incidence of lesions on 7 year-old 'Ray Ruby' grapefruit trees, Ft. Pierce, FL, USA.

3 RESULTS AND DISCUSSION

The size, morphology and dispersion of CS-CuPs were observed through electron microscopy and DLS analysis. The silica core particle was found to be spherical, sub-micron (350-600 nm) in size and well dispersed (**Figure 1, Panel A**). The size of the particles were determined using ImageJ software analysis of at least 4 separate SEM images (not shown) and a size distribution with Gaussian curve was produced (**Figure 1, Panel A inset**). In contrast to the coated SiP core, the CS-CuP particle was found to be semi-spherical, sub-micron (350-600 nm) in size and less dispersed due to neutralization of surface charge at neutral pH (**Figure 1, Panel B**). The size of the particles were determined using ImageJ software analysis of at least 4 separate SEM images (not shown) and a size distribution with Gaussian curve was produced (**Figure 1, Panel B inset**). SEM results confirmed the increase in the average particle size and size distribution after coating the silica core with the Cu-silica gel layer. HRTEM image further confirmed the formation of Cu-silica gel layer deposition over the silica core. A clear difference in contrast between the core and the shell was noticed (**Figure 1, Panel C**). The Cu-silica shell thickness appears to be uneven with varying thickness between ~20 – 40 nm. DLS measurements further confirmed particle size and size distribution increase of the SiP core after coating with a Cu-silica gel layer (**Figure 1, Panel D**).

The *in-vitro* antimicrobial efficacy of the CS-CuPs was evaluated by determining the MIC against multiple model phytopathogens. Efficacy of CS-CuPs were comparable to (or in some cases slightly better than) the controls, copper hydroxide and copper sulfate (MIC results are shown in **Table 1**). Our *in-vitro* efficacy results prompted to pursue further efficacy evaluation in field conditions. In the 2014 citrus canker trial (conducted in Fort Pierce, FL on "Ray Ruby" grapefruit), it was clearly seen that CS-CuPs demonstrated strong efficacy over copper hydroxide, copper oxide and copper sulfate commercial standards **(Table 2)**. CS-CuPs were able to reduce infection to 15.6% from 62.8 %, compared to copper hydroxide (16.4 %), copper oxide (16.0%) and copper sulfate (18.4 %) while being applied at a significantly lower metallic Cu rate **(Table 2).** Our results confirm the hypothesis that core-shell particle design is effective in maintaining efficacy against canker infection at lower application rate. The present CS-CuP particle formulation contains 25,450 µg/mL metallic Cu and the process is scalable.

4 ACKNOWLEDGEMENTS

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