Copper nanomaterials against copper-tolerant strains of *Xanthomonas perforans* and bacterial spot of tomato

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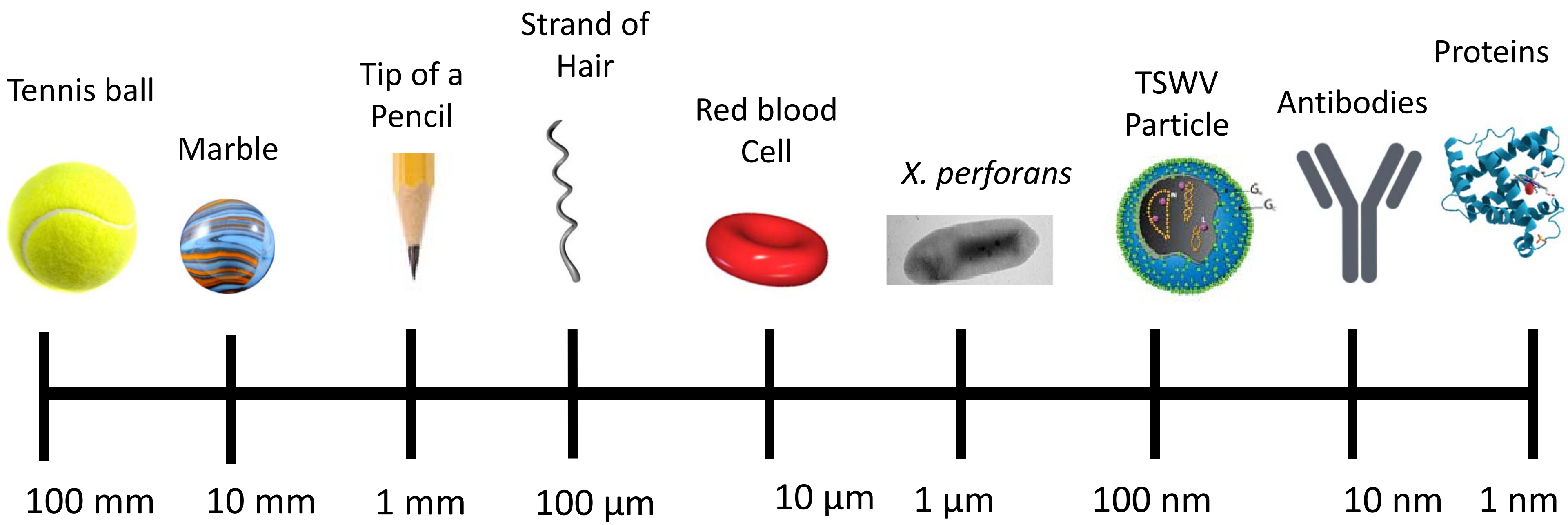
- Copper-tolerance?
  - **Nanoparticles vs. micron counterparts**: antibacterial activity of metallic compounds is size dependent
    - Smaller particles with larger surface to volume ratios have more activity
    - Interact more closely with microbes
    - Releases more metal ions in solution

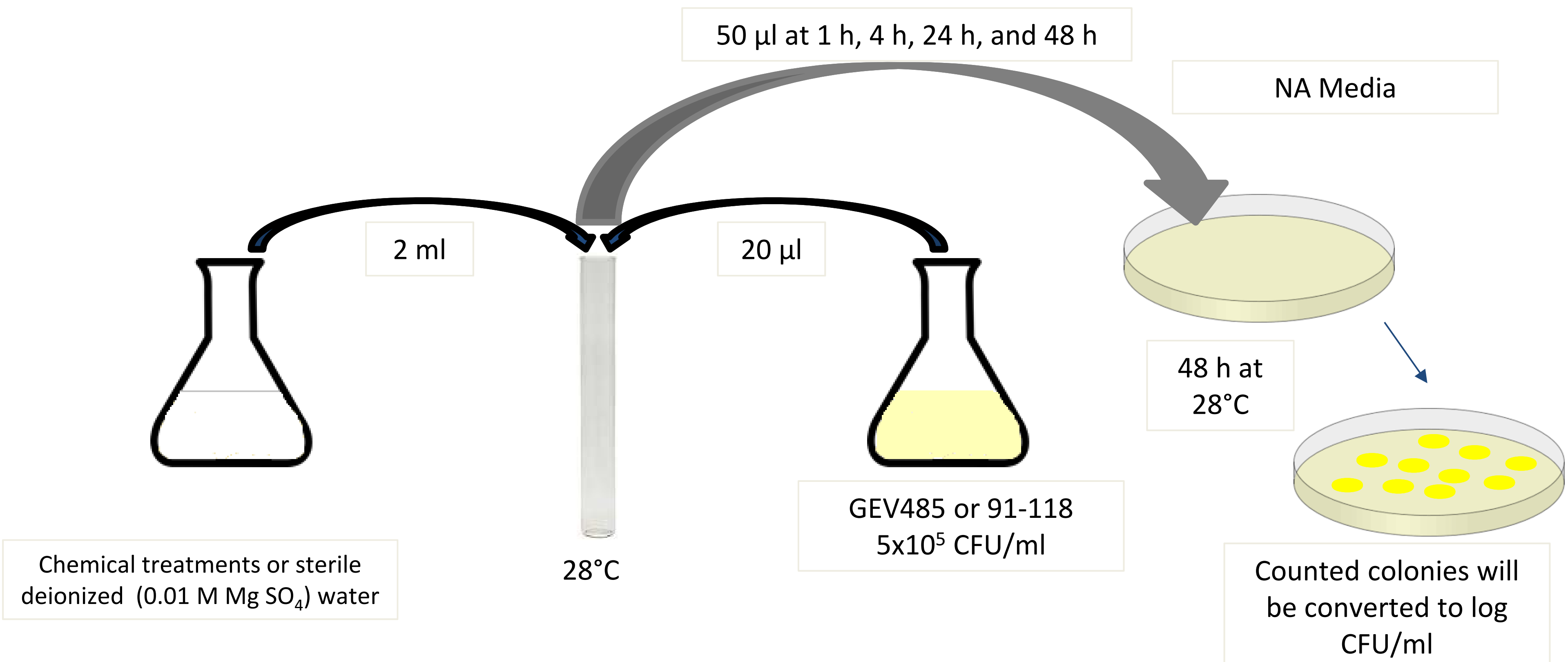
Hypothesis: Reducing the size of Cu to nanosize form and unique structural modifications will improve antibacterial properties when compared to micron size particles

Nanometer?



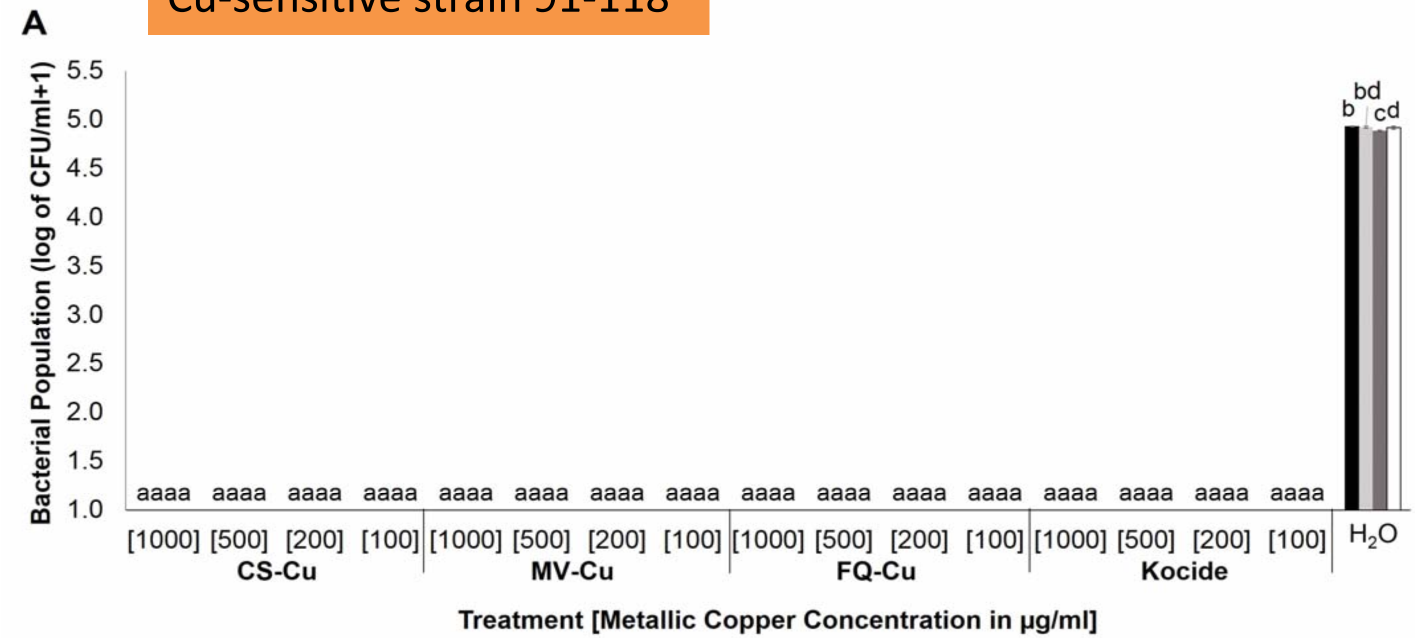
Photos courtesy of Ocsoy et al. 2013, Phillips et al. 1980, and Sherwood et al. 2003.

- GEV485 (Cu-tolerant) and 91-118 (Cu-sensitive) *X. perforans* strains were grown on NA for 24 h and then transferred to NA amended with 20 µg/ml of Cu ($CuSO_4 \cdot 5H_2O$) for 24 h
- Treatments:
  - 100, 200, 500 and 1000 µg/ml of metallic copper from CS-Cu, MV-Cu, Cu-FQ, and Kocide 3000
  - Deionized water amended with 0.01 M $MgSO_4$ (nontreated control)

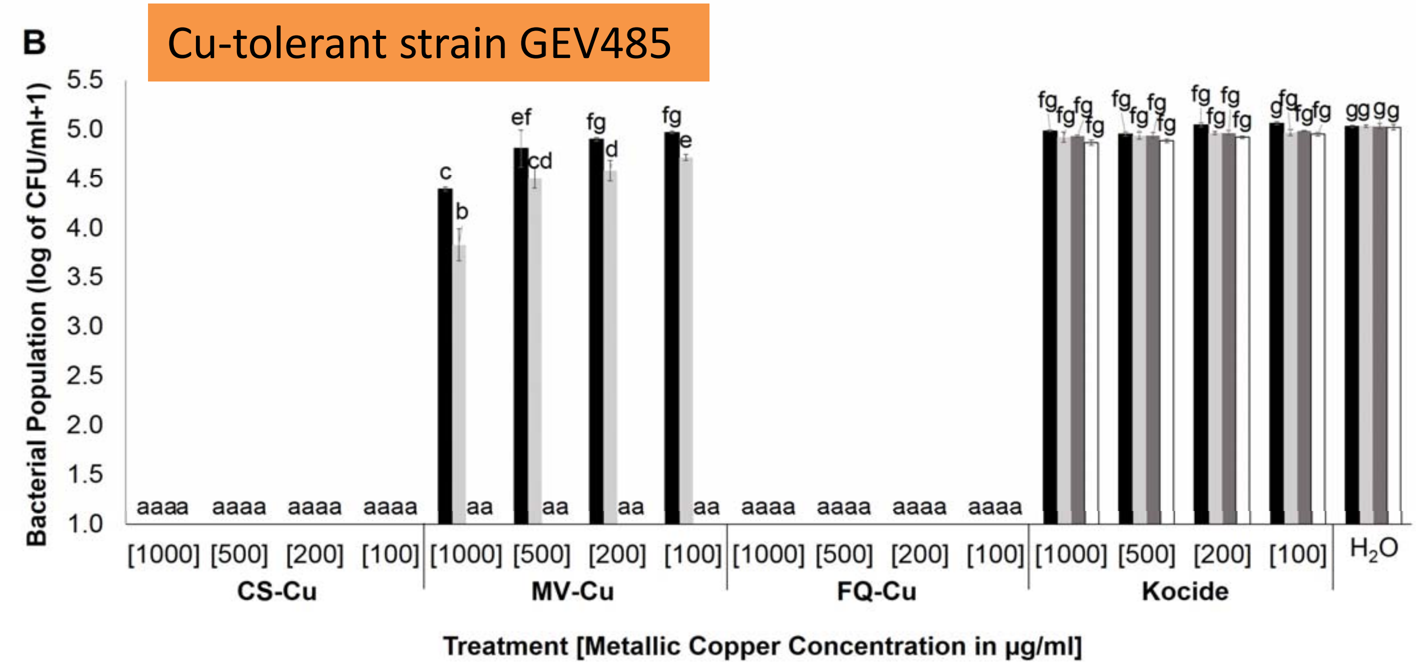


In vitro Results

Cu-sensitive strain 91-118



Cu-tolerant strain GEV485



In vitro activity of Core shell silica copper, CS-Cu; Multivalent Copper, MV-Cu; and Fixed quaternary ammonium copper, FQ-Cu) and Kocide® 3000 (DuPont™, Wilmington, DE) on *Xanthomonas perforans* survival over time (**Black bars=1 h, Light Gray bars=4 h, Dark Gray bars=24h, and White bars=48 h**). **A,** 91-118 (Cu-sensitive strain). **B,** GEV485 (Cu-tolerant strain)

Seed germination

21 days

Foliar Treatments (4 plants per treatment)

75, 100, 200, 500, and/or 1000 µg/ml of metallic copper from CS-Cu, MV-Cu, Cu-FQ, and DuPont™ Kocide® 3000
DuPont™ Kocide® 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L)
Water treated control

24 h

Spray inoculated with GEV485 (Cu-tolerant) suspension at 5 x 10^8 CFU/ml

28°C

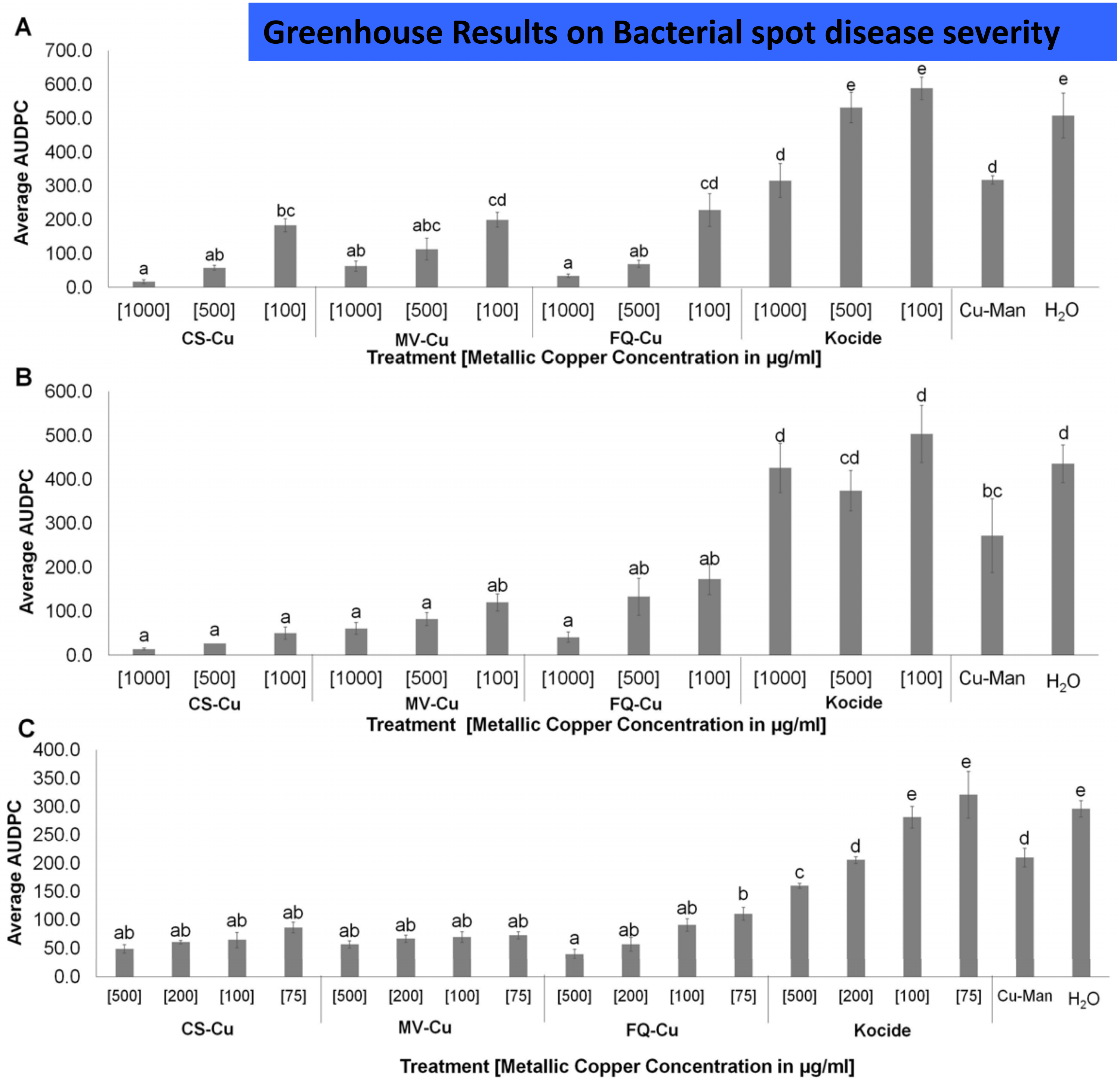
48 h

28°C

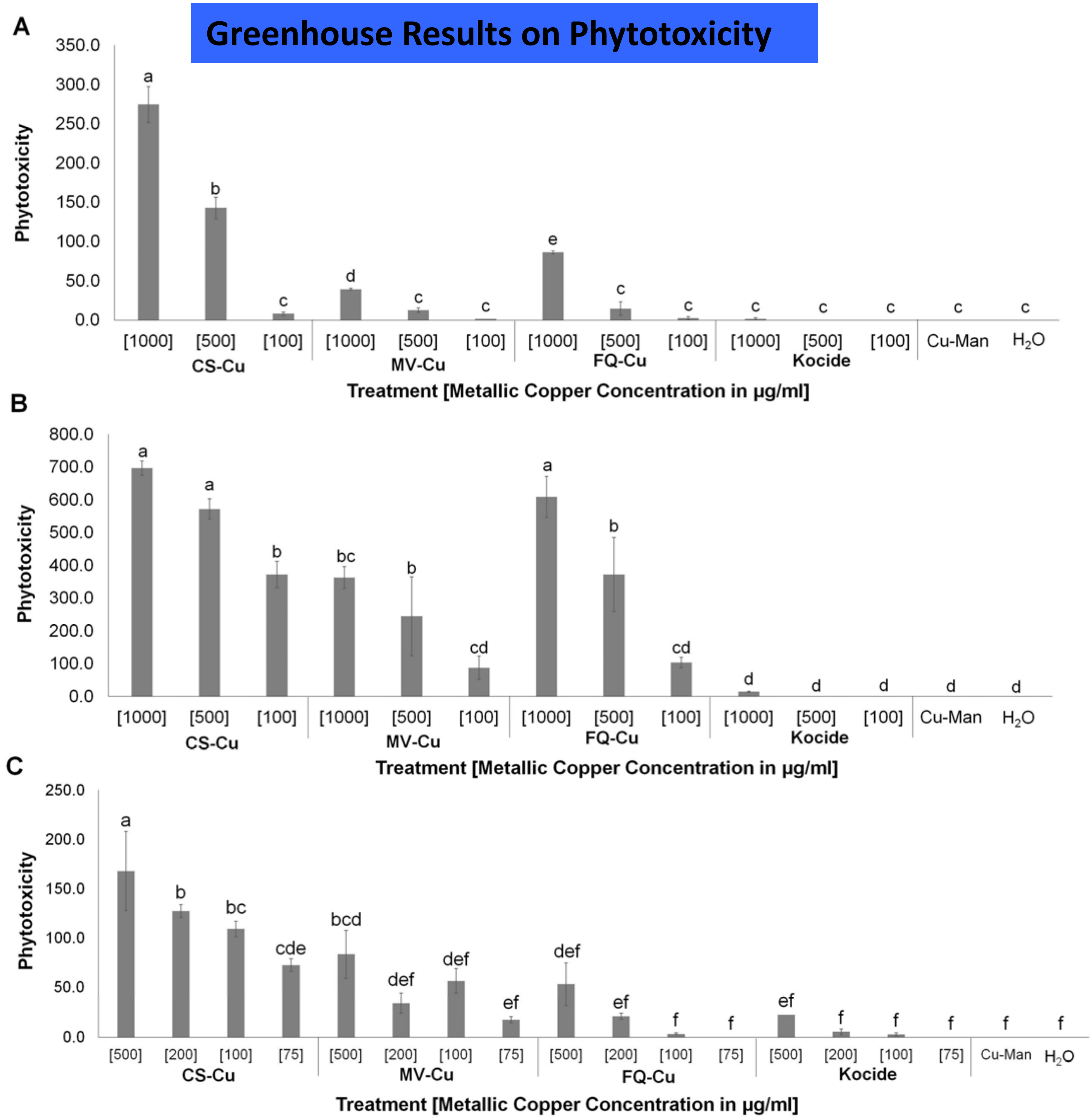
3 days

Beginning at 5 days post inoculation and until 16 days, plants were rated using the Horsfall-Barratt (1945) disease severity scale and AUDPC was calculated as previously described (Madden 2007)

Greenhouse Results on Bacterial spot disease severity



Greenhouse Results on Phytotoxicity



- Fall 2015 (Quincy, FL) and Spring 2016 Field Trials (Wimauma and Quincy, FL)
  - 4 plots/treatment
  - 15 Plants (BHN602)/plot (Quincy, FL) and 10 Plants (HM1823)/plot (Wimauma, FL)
  - Complete Randomized Block Design
- Treatment List:
  - 100 and 200 µg/ml of metallic copper from CS-Cu, MV-Cu, Cu-FQ
  - DuPont™ Kocide® 3000 1.75 lb/a
  - DuPont™ Kocide® 3000 (1.75 lb/a) and Penncozeb® 75DF (1 lb/a)
  - Water
- Field applications of treatments:
  - 1 week prior to inoculation
  - Up to 9 weekly applications post inoculation
- Plants were inoculated with a suspension of GEV485 (5 x10^8 CFU/mL):
  - The two end plants and the middle plant in each plot were inoculated
- Disease and phytotoxicity were assessed using the Horsfall-Barratt (1945) disease severity scale and the midpoint of the percent ranges were used to calculate the area under disease progress curve (AUDPC) (Madden et al. 2007).

Field Results on Bacterial spot disease severity

	Fall 2015 Quincy, FL		Spring 2016 Wimauma, FL		Spring 2016 Quincy, FL	
Treatment [Metallic Copper Concentration]	**AUDPC[z]**	**Total[y]**	**AUDPC[z]**	**Total[y]**	**AUDPC[z]**	**Total[y]**
CS-Cu [100 µg/ml]	598.9 ab	68,616 a	778.6ab	29,626 a	836.9 a	21,526 a
CS-Cu [200 µg/ml]	588.3 ab	74,291 a	687.4a	33,139 a	837.8 a	22,961 a
MV-Cu [100 µg/ml]	479.5 a	62,598 a	727.6a	28,249 a	887.0 a	25,401 a
MV-Cu [200 µg/ml]	538.4 a	57,185 a	832.6ab	27,190 a	832.4 a	23,822 a
FQ-Cu [100 µg/ml]	589.9 ab	53,137 a	713.4a	35,945 a	868.6 a	23,965 a
FQ-Cu [200 µg/ml]	662.4 ab	54,836 a	689.9a	35,424 a	938.4 a	22,243 a
Kocide [540 µg/ml]	671.4 ab	61,025 a	972.1ab	32,168 a	1135.4 ab	22,387 a
Cu-Man [540 µg/ml]	595.9 ab	53,430 a	773.4ab	32,062 a	1188.0 ab	19,229 a
H_2O	773.5 b	60,113 a	1136.8b	28,691 a	1402.1 b	22,100 a

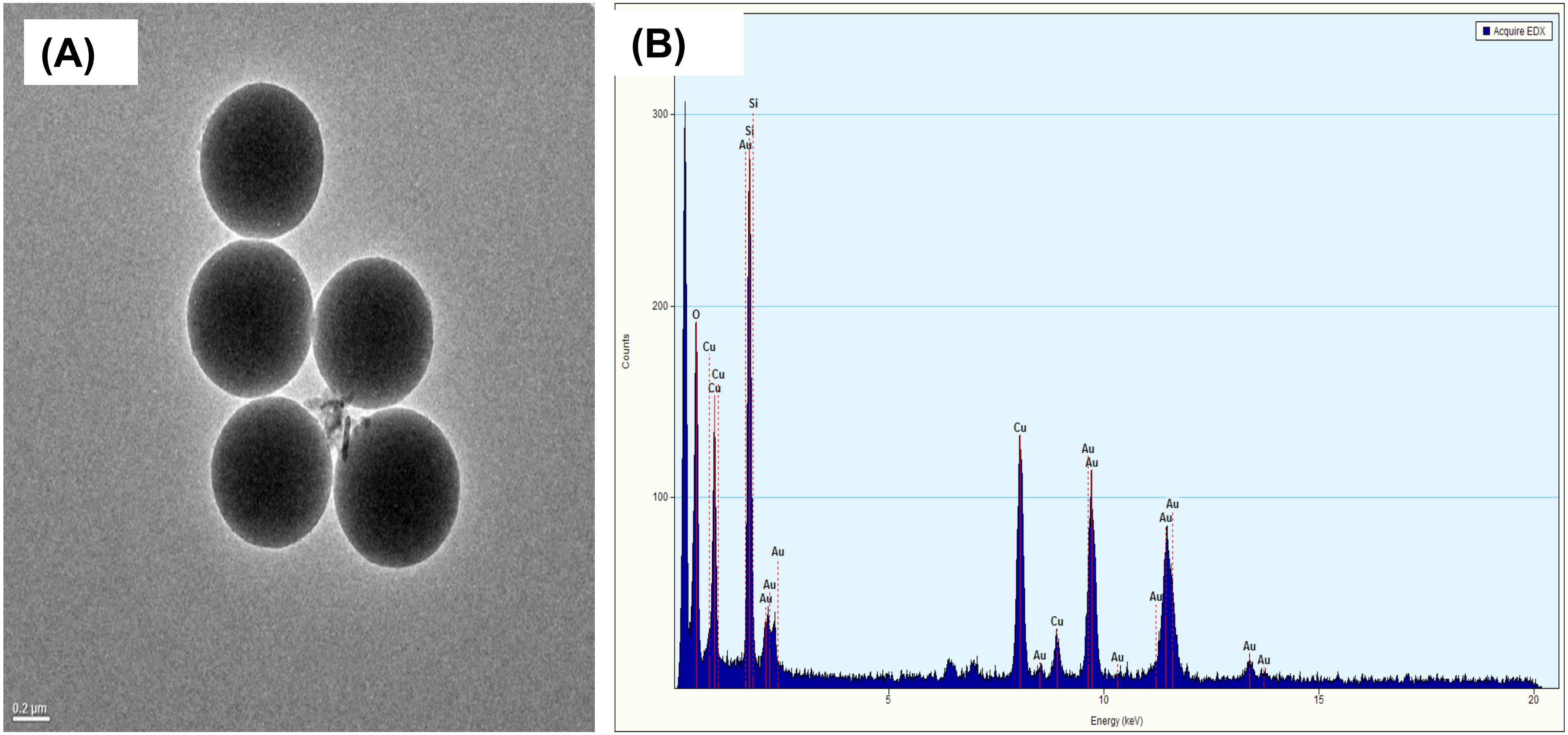
SNK Analysis with a P=0.05

*No phytotoxicity was observed in any of the field experiments (data not shown)

Summary

- All of the copper-based nanomaterials had greater antibacterial activity against copper-tolerant strains *in vitro* when compared to metallic copper from Kocide 3000
- In the greenhouse, most of the copper-based nanomaterial concentrations significantly reduced bacterial spot disease severity when compared to Copper-Mancozeb
- In the field, all of the copper-based nanomaterials reduced bacterial spot disease severity when compared to the untreated control
  - Differences in yield were not observed
  - Phytotoxicity was only observed under greenhouse conditions
- Current Experiments
  - The effect of copper-nanomaterials on the bacterial population in the soil in collaboration with Dr. Jason Hong from USDA-ARS in Ft. Pierce Florida.
  - Effect of Integration of Cu nanomaterials with SARs and biocontrol agents

High-Resolution Transmission Electron Microscopy (HRTEM) of Core-Shell Cu (Cu-CS)

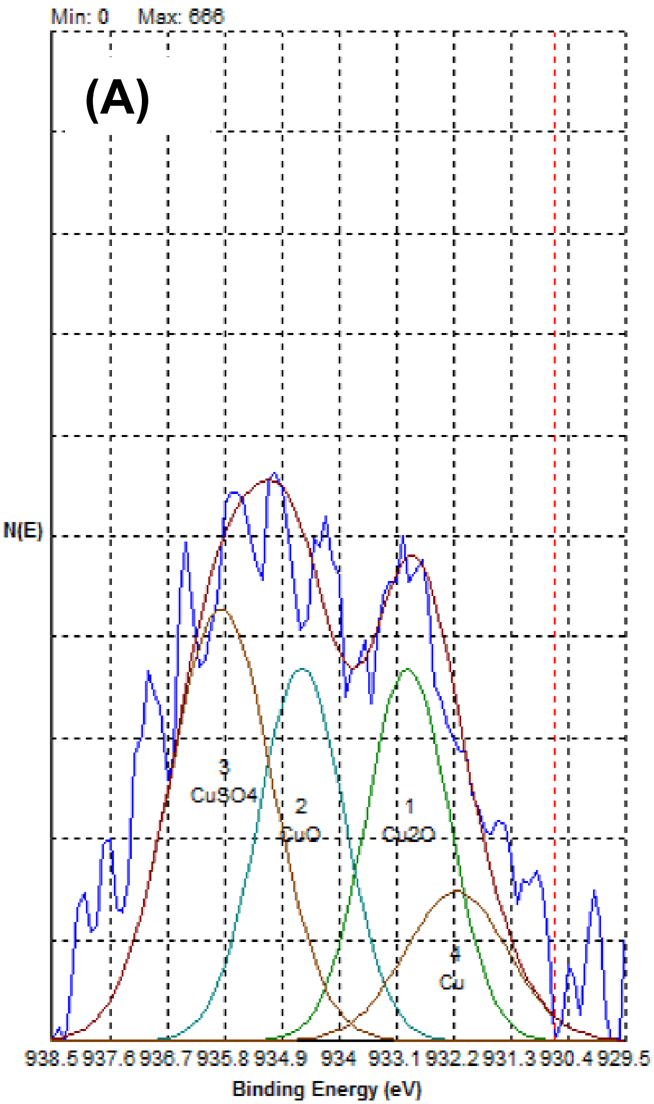


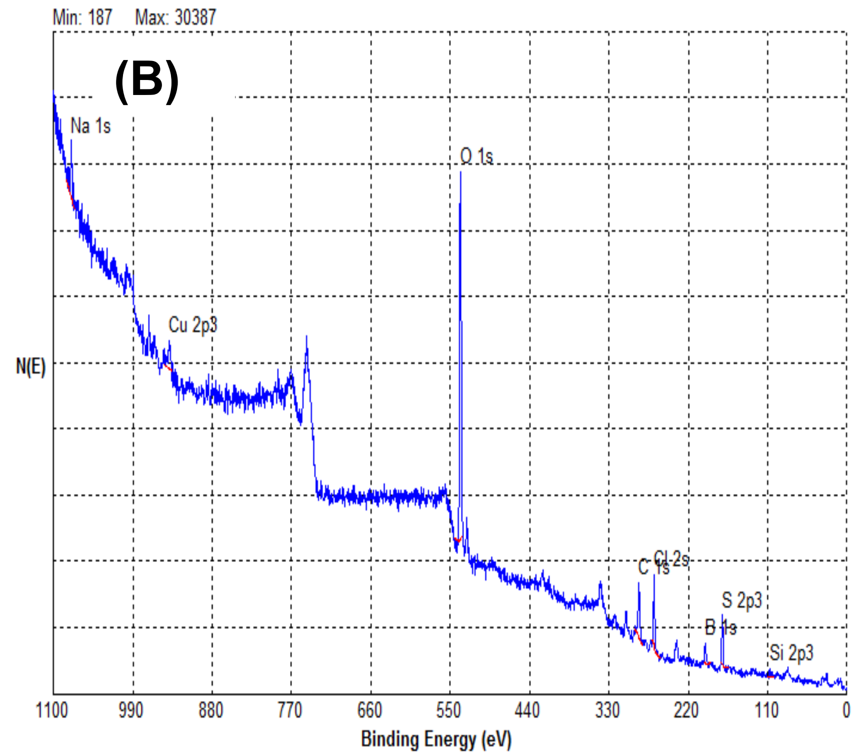
Images of Cu-CS with scattered dark contrast confirming presence of electron-rich material.

(A) Particles are sub-micron in size varying between 300-600 nm.

(B) Energy dispersive spectroscopy of Cu-CS particles showing the silica present in the core of the particle

X-ray Photoelectron Spectroscopy (XPS) of Cu-MV: Signature of Cu with multiple valence states





(A) High resolution spectra of Cu in Cu-MV revealing metallic Cu, copper (I) oxide, copper (II) oxide and copper sulfate.

(B) Survey spectra of Cu-MV

Developing a commercially-viable Cu formulation

Challenges and commercial viability

- Selection of suitable inerts for delivering actives – EPA approved or GRAS-category
- Transition from chemical-grade to agri-grade chemicals – should maintain efficacy and safety
- Residual activity – comparable or reduced level to current industry standards
- Formulation development process (number of steps) – simple, # of steps should be minimized
- Cost of raw materials (agriculture – grade) – cheap!
- Shelf-life (~2 years)

University of Florida Opportunity Seed Grant, 2011-2013

IFAS Early Career Scientist Award, 2012-2014

2013
2016

USDA-SCRI (2015-51181-24312) 2015-2019

United States Department of Agriculture
National Institute of Food and Agriculture

USDA-AFRI Nanotechnology in Agriculture (2016-67021-24992) 2016-2020

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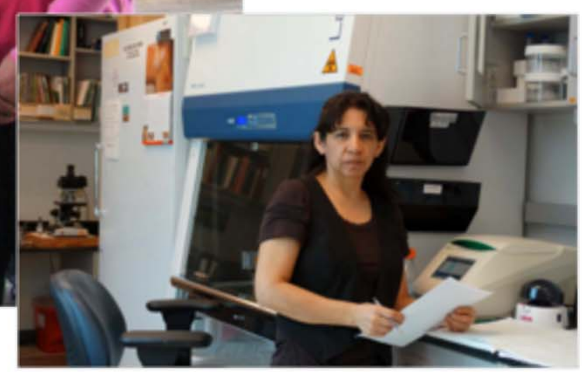


Our Lab Info

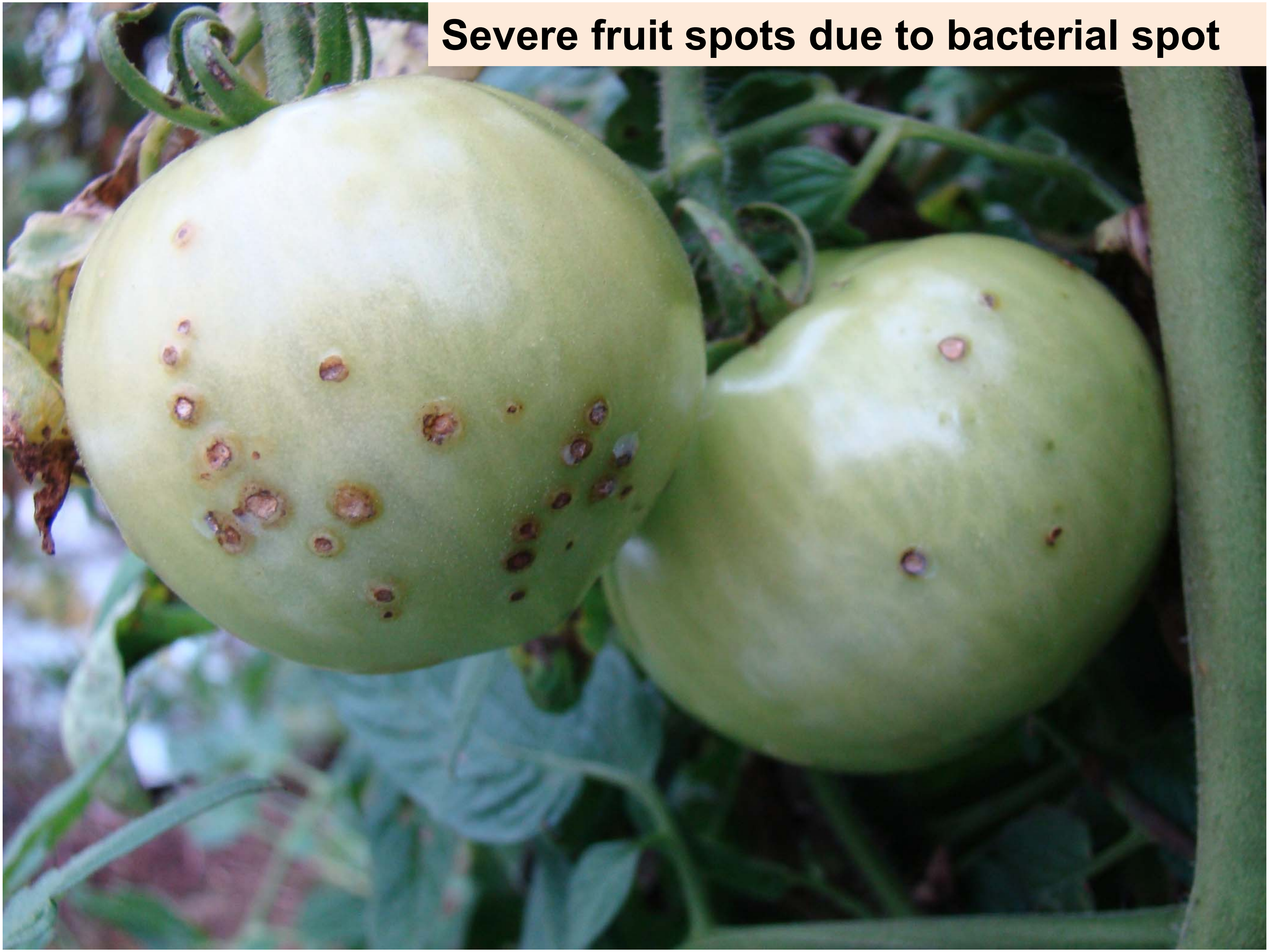
This picture shows what a FL tomato grower faces during critical production periods through the year

Bacterial Spot of Tomato

Shot-hole like appearance of the lesions on the leaves

Severe leaf spots due to bacterial spot

Severe fruit spots due to bacterial spot



Severe defoliation and fruit infections can cause 20-50% yield losses

Bacterial Spot of Tomato

- First discovered in South Africa in 1914
- Caused by four distinct species of ***Xanthomonas*** (*X. euvesicatoria, X. gardneri, X. perforans,* and *X. vesicatoria*)
- As of 2006, *X. perforans* is the dominant species in Florida.
  - Antibiotics was in use 1950s; continuous field use led to bacterial resistance development. Currently only used in transplant production.
  - Current Practices: Pathogen free seed and clean transplants
  - **Use of Copper + EBDC (e.g. Mancozeb) (++)**
    - As of 2006, all *X. perforans* strains (375+) in Florida are copper-tolerant. **Copper (-)**.

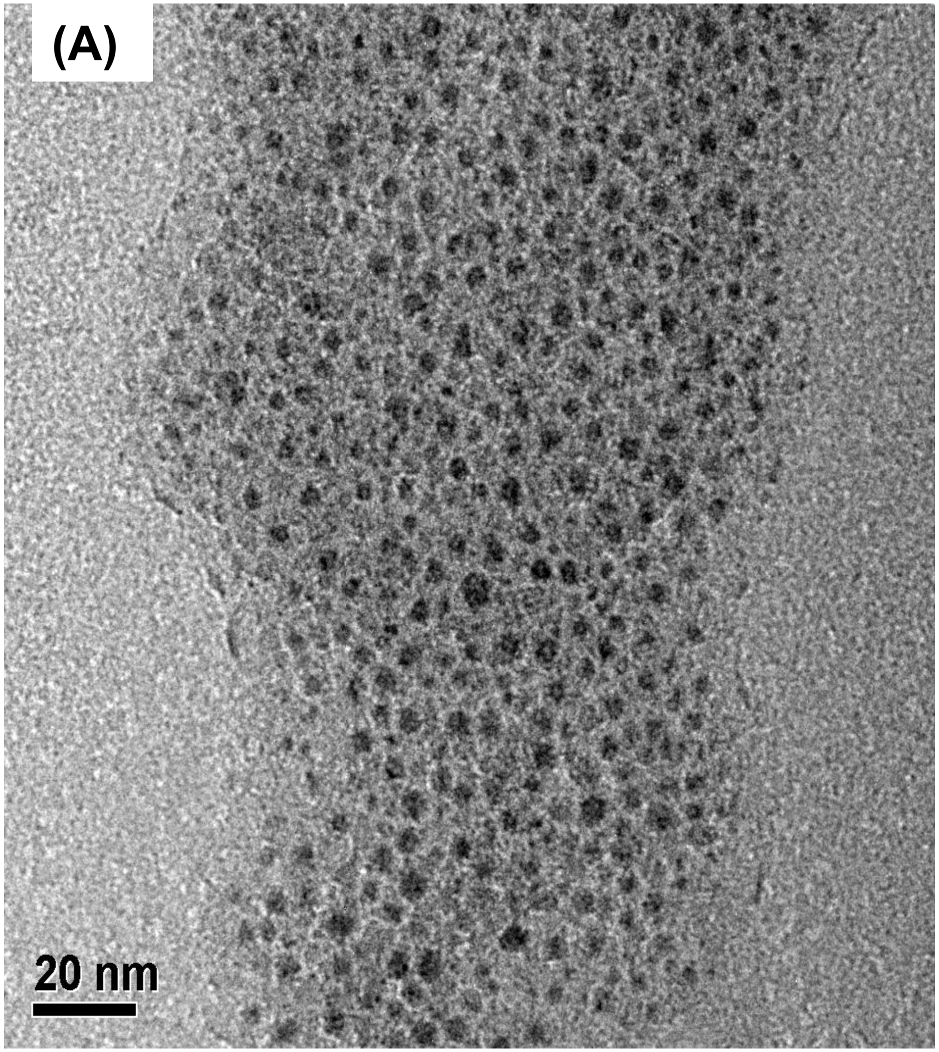
– Other materials: **SAR inducer (Actigard; ++)**, **bacteriophages (+/-)**, **biocontrol agents (+/-)**

– Limited options necessitates development of new approaches

Cu - nanocomposite characteristics

- 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 of water-soluble Cu compounds (such Cu salts and Cu chelates) is high, however, they exhibit phytotoxicity.
- To address these limitations, mixed valence (MV) Cu loaded silica nanogel and core-shell (C-S) Cu nanoparticle materials was developed.
- It is hypothesized that MV Cu system, specifically enriched with Cu (0) and Cu (I) will exhibit enhanced antimicrobial efficacy over traditional Cu (II) compounds.
- Materials under development:
  - Cu-CS (Copper Core-shell particle)
  - Cu-MV (Copper Mixed-valence gel)

High-resolution Transmission Electron Microscopy
Copper Mixed-Valence (Cu-MV)

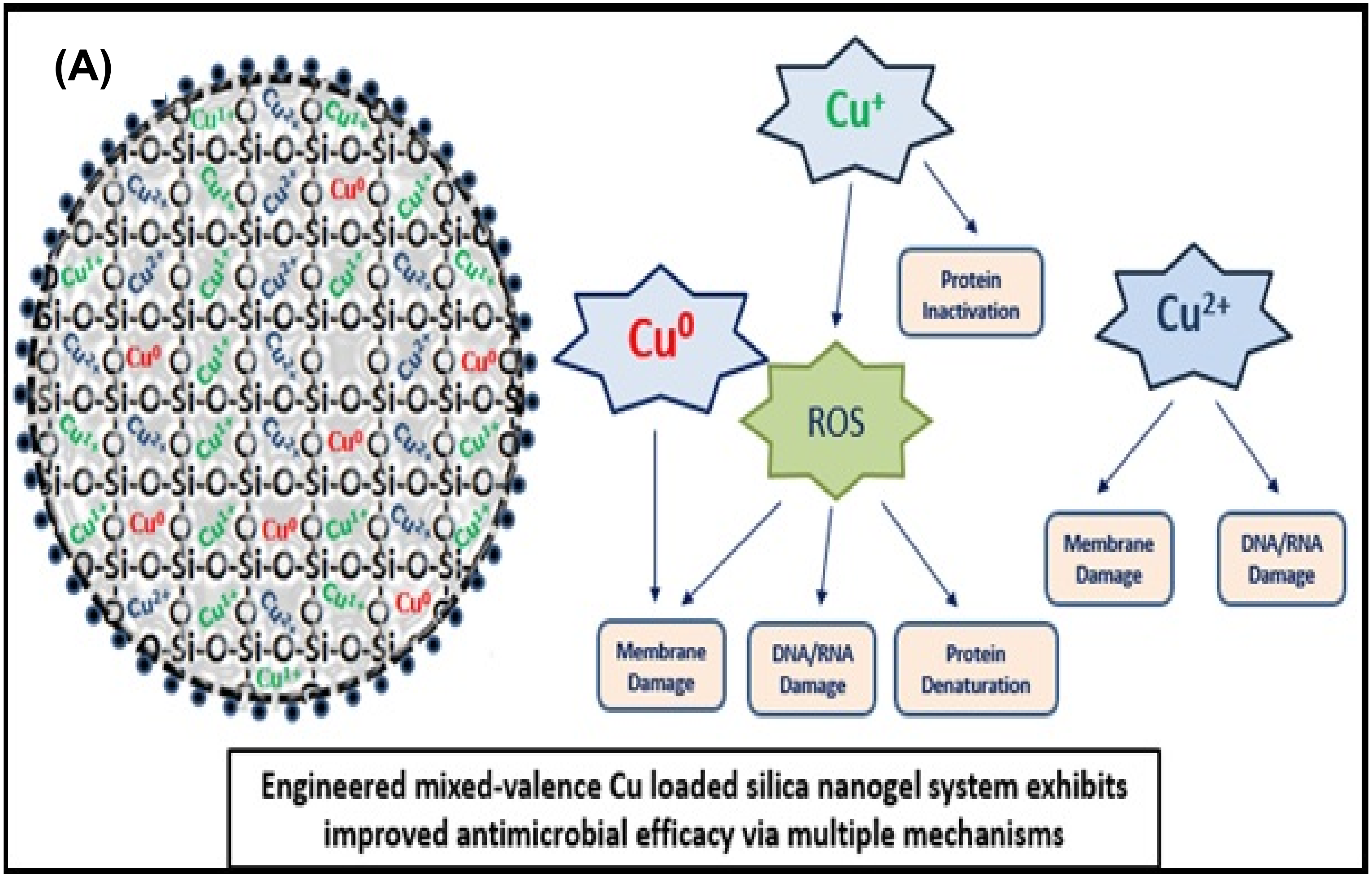


Low magnification image of Cu-MV with scattered dark contrast confirming presence of electron-rich material.

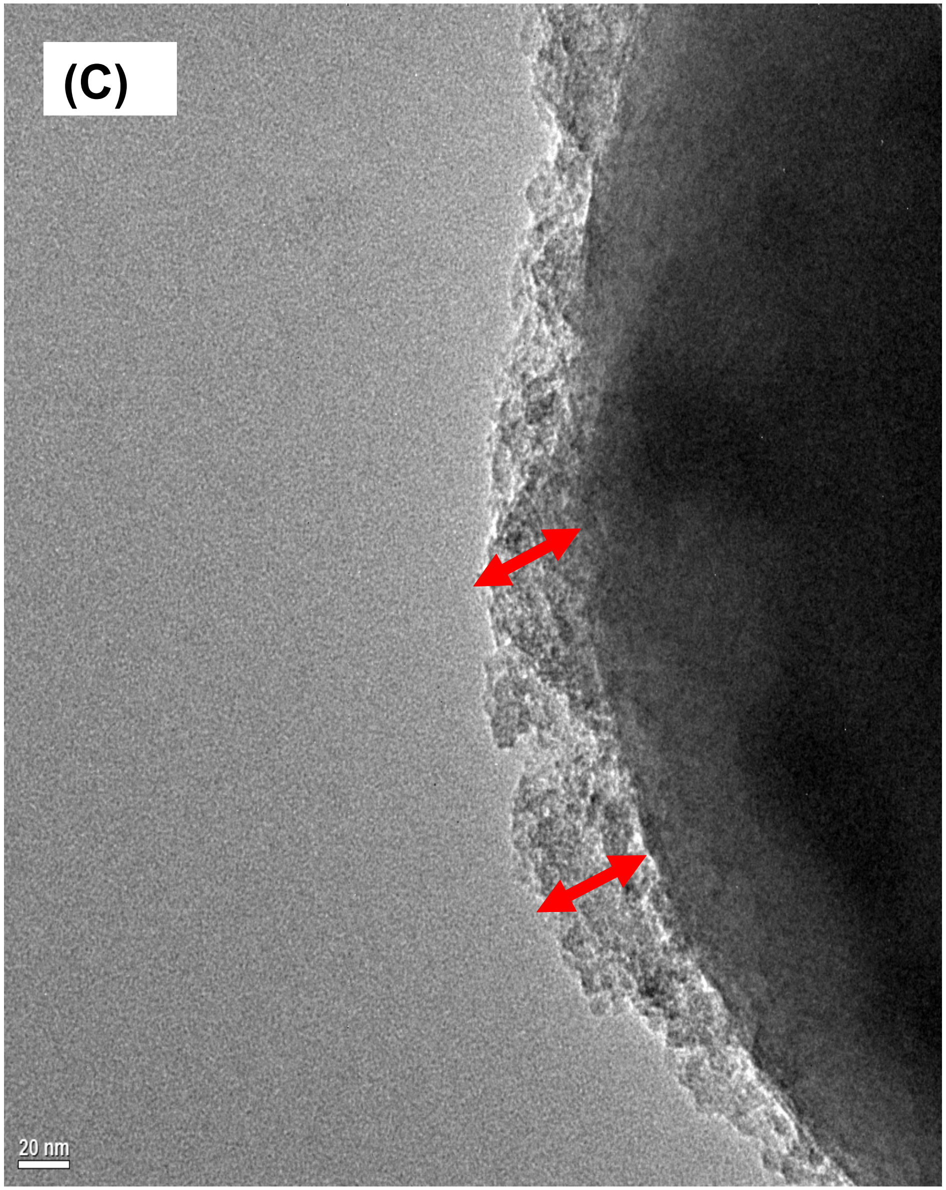
(A) Particles are interconnected in a large micron sized gel matrix

Micron size gel matrix embedded with nano-size copper actives creates a well dispersed film of coverage for crop protection

Mixed-Valence Cu loaded silica nanoparticle



(A) Brief summary of biocidal mechanism of action of Cu oxidation states



Cu-CS HRTEM

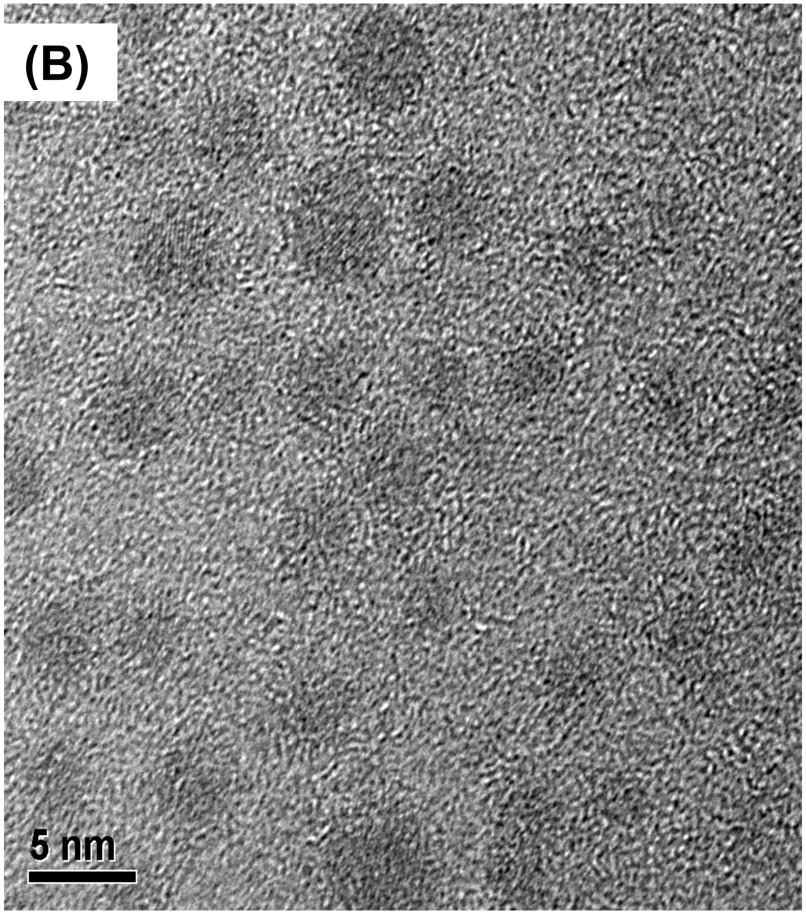
Difference in contrast demonstrates variance in core and shell materials

Compound	Lattice spacing (Å)
Copper (II) oxide	2.32, 1.87
Copper (I) oxide	2.47, 3.02

- **Calculated lattice spacing in Cu crystallites reveal multiple Cu oxidation states present in particle shell**
- **Absence of Cu in the core reduces unavailable Cu, thereby increasing Cu bioavailability and efficacy**

(C) Difference in contrast and crystallinity confirm the presence of shell 30-50 nm

High-resolution Transmission Electron Microscopy
Copper Mixed-Valence (Cu-MV)



Compound	Lattice spacing (Å)
Copper (II) oxide	2.32
Copper (I) oxide	3.02, 2.47
Cu	2.09

Calculated lattice spacing in Cu crystallites reveal multiple Cu oxidation states present in Cu silica gel

agnification image of Cu-MV with Individual Cu crystallites seen as 3-8 nm in size