

Synthesis and Evaluation of Copper Oleate Nanoparticles Against Citrus Anthracnose Caused by *Colletotrichum gloeosporioides*: In Situ Experiments, In Vitro Bioassays, and Field Trials

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Anthracnose in citrus is a severe disease caused by *Colletotrichum* species. Synthetic fungicides have been used to combat this citrus disease; however, they may have adverse health effects on humans and cause harmful environmental impacts. During a search for environmentally friendly fungicides, we successfully prepared organic copper (copper (II) oleate) nanoparticles by using natural chitosan polymer as a coating agent and stabilizer and by combining an exchange reaction and *in situ* coating method. Organic copper nanoparticles coated with chitosan polymer (Cu-oleate@CS NPs) with Cu contents ranged from 5.0 to 6.0% by weight. The nanoparticles have an average particle size ranging from 50 nm to 60 nm (TEM), covered by a layer of polymer with a thickness of about 10 to

12 nm. The analytical results of zeta potential and dynamic solution size showed that the nanoparticles are uniformly dispersed (90–120 nm by DLS), with high stability (zeta potential = −61.7 mV) and low cytotoxicity to Vero healthy cell line. Cu-oleate@CS NPs significantly inhibited the mycelial growth of *C. gloeosporioides* *in vitro* at 500 and 1000 µg/mL. In field trials, the best antifungal efficacy of Cu-oleate@CS NPs against citrus anthracnose on orange plants was found at a spray concentration of 0.33% with a disease control efficacy of 54.18% at 14 days after the second spraying. Our study results demonstrated a novel preparation of Cu-oleate@CS NPs and suggested that it could be used as a promising nanoformulation to control citrus anthracnose *in vitro* and *in vivo*.

Introduction

Chemical pesticides are considered a powerful weapon for humans in preventing pests and protecting crops. However, the excess amount of chemical pesticides during the cultivation is the perpetrator causing many other serious harms such as pollution of the soil and air environment, especially the water environment due to leaching leading to serious surface water pollution. Besides, residues of chemical pesticides are also toxic to humans and livestock, directly affecting consumer health. The negative effects of pesticides become more serious and are alarming when people use them incorrectly and overuse them. This not only reduces biodiversity and harms natural enemy populations, but also gives rise to drug resistance in pests and

diseases, increases disease prevention costs, and greatly affects human health and the environment. Therefore, finding an effective and safe pesticide for humans and warm-blooded animals is an extremely urgent issue to build sustainable agriculture.

Currently, copper-based pesticides have been widely used in crop protection. They have good effects on plant disease control and contribute to improving crop productivity.^[1–4] However, current copper-based active ingredient pesticides are mostly inorganic compounds including main groups such as copper oxychloride, copper hydroxide, and copper sulfate, belonging to toxic group II, which are decomposed slowly in the environment and are especially easy to wash away after spraying, so the actual dosage is very large.^[1,4] In sustainable agriculture practices, Europe and some developed countries are going to ban inorganic copper-based pesticides and replace them with green pesticides.^[5–10]

Current research on the organic copper form has been mainly looking for methods to synthesize copper complexes with organic acid ligands, and focus on improvement of biological activity. However, this leads to a cause of higher toxicity and high risks compared to copper ions in inorganic salts.^[7,11–15] Many published patents focused on preparation of organic copper compounds to minimize environmental impact. For example, forms of organic copper compounds were described in patents EP2123161 A3 on “Product for agricultural use based on organic copper complexes” and US3323987A on

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“Fungicidal preparations containing copper bis-valerianate”^[16,17] The organic copper compounds described in these patents were all chelated complexes of copper with organic acids. The organic coppers with lower copper contents allow for preventing or minimizing the attack of various phytopathogenic fungi or bacteria on crops and consequently cause less environmental impact. In addition, organic copper also induces and boots the plant defense system, promoting plant vitality and growth, and thus leading to larger crop productivity with higher quality. US Patent 2005/0159482A1 “Biologically activity copper organic agents ” provides a fungicide and bactericide based on organic copper salts in the form of chelated complexes, these compounds have the formula $\text{Cu}^{2+} \cdot 2\text{R}$ with a 1:2 ratio between copper and organic radicals, whereby this radical has the form $\text{O}^{-}-\text{CO}-\text{CH}(\text{SH})-\text{CH}_3$.^[14] However, these resulting products have the problem of not significantly reducing the amount of copper applied per hectare compared to the long-used inorganic copper.

Nanomaterials have emerged as a cutting-edge innovation in all kinds of industrial sectors.^[18] Nanomaterials plays a vital role and has many applications in the fields of medicine,^[19–22] biodefence, agriculture along with agro-products,^[23–27] food industries,^[28] biotechnology,^[23] environmental pollution detection and control^[29,30] water treatment,^[31,32] and electronic,^[33,34] due to various physicochemical properties of nanomaterials in comparison to microscale and bulk materials. These unique properties are naturally due to elevated atoms fraction and surface energy of miniature particles in comparison to bulk materials. The employing of nanotechnology in agriculture is considered as a new direction to develop a more effective, economical, and safer agriculture. Generally, active pesticide ingredients are poorly soluble in water, making their use difficult, so nanomaterials can increase the water solubility of the main active ingredients. In this way, the release and distribution of the pesticides can be better controlled and the active substance can be protected from too rapid degradation. Not only does this improve the absorption and pesticide effectiveness, it also significantly reduces the amount of pesticides applied in the field. Furthermore, the nano forms of active ingredients and pesticides may intend to be less harmful to users, environmentally friendly, and may reduce the development of pest resistance.^[24,37,38] Nanotechnology has been applied to prevent and treat diseases caused by bacteria, viruses, and fungi on plants, and animals, and to preserve agricultural products. Micronutrient nanoparticles also act as a biological stimulant. In crop protection, the nanoparticles of active ingredients such as nanosilvers and nanocoppers have been researched to be fungicides and for controlling fungal plant diseases.

According to Choudhary et al. (2017), Cu-chitosan nanoparticles can enhance defense responses against leaf spot diseases and plant growth in maize (*Zea mays* L.).^[35] This is an important development in agricultural nanomaterials research, biodegradable Cu-chitosan nanoparticles being more compatible with biological control because these nanoparticles “copy” the natural adaptation of plant defense and antioxidant systems to protect plants. The authors Saharan and colleagues (2015) in

a research synthesized and evaluated the antifungal effect of *Solanum lycopersicum* Mill in in vitro experiments of Cu-chitosan nanoparticles.^[36] Laboratory-synthesized nanoparticles showed significant growth-promoting effects on tomato seed germination and seedling length. At a concentration of 0.12%, these nanoparticles inhibited 73.5% of mycelium growth and 61.5 and 83.0% of germination inhibition of spore in *Alternaria solani* and *Fusarium oxysporum*.^[36] However, this formulation was still inorganic, with free copper nanoparticles coated with polymers such as chitosan. Meanwhile, research on nanoforms of organic copper salt compounds for agricultural applications is still very new, and there are no in vivo tests to evaluate their effects on plants and phytopathogenic fungi. Therefore, there is a high demand for a technological method to create a new nano-based product of organic copper salts to prevent plant diseases, thereby helping farmers reduce the use of synthetic pesticides during crop cultivation.

In this research, we present a method to synthesize nanoparticles of copper II oleate product (Cu-oleate@CS NPs) coated in natural chitosan, which has good biodegradability and biological activity to enhance the bioefficacy and facility the use in high-tech and sustainable agriculture. We described a novel approach to the fabrication of nanoparticles by using the exchange reaction from salts of fatty organic acids with inorganic copper salts dispersed in chitosan solution through complexation with $-\text{NH}_2$ and $-\text{OH}$ groups of chitosan, and combined with an in-situ coating process (coating immediately during nanometer-sized particle formation). Cu-oleate@CS NPs obtained in this study were tested for its *in vitro* antifungal activity against *C. gloeosporioides*, a causal agent of citrus anthracnose.^[39,40] Additionally, the nanoformulations containing Cu-oleate@CS NPs were employed to field trials on orange trees to evaluate their disease control efficacy against citrus anthracnose.

Results and Discussion

Characterization of Cu-Oleate@CS NPs

The Cu-oleate@CS NPs were obtained by the *in-situ* method in a turquoise liquid form.

The measurement results of infrared absorption spectroscopy of Cu-oleate@CS NPs are presented in Figure 1a. The results show that the IR spectrum of Cu-oleate@CS NPs has characteristic absorption bands of the stretching vibrations of the $-\text{OH}$ group at 3415 cm^{-1} , the ether $-\text{O}-$ group at 1086 cm^{-1} , the $\text{C}=\text{O}$ stretching vibrations at 1712 cm^{-1} of the acetyl group of chitosan, two bands at 1605 and 1414 cm^{-1} were assigned to stretching vibrations of COO^- group of oleate. Their $\Delta\nu\text{COO}$ value of 191 cm^{-1} has an increase by 56 cm^{-1} in comparison with that value in free oleate ion, showing a significant interaction with Cu^{2+} .^[41,42] The absorption bands in the region of $2800\text{--}3000\text{ cm}^{-1}$ is due to the asymmetric ν_{as} and symmetric ν_{s} stretching vibrations of the methyl (CH_3) and methylene (CH_2) groups of the hydrocarbon radical.^[41] The absorption band of the $-\text{NH}_2$ bending vibration of chitosan appearing at 1599 cm^{-1}

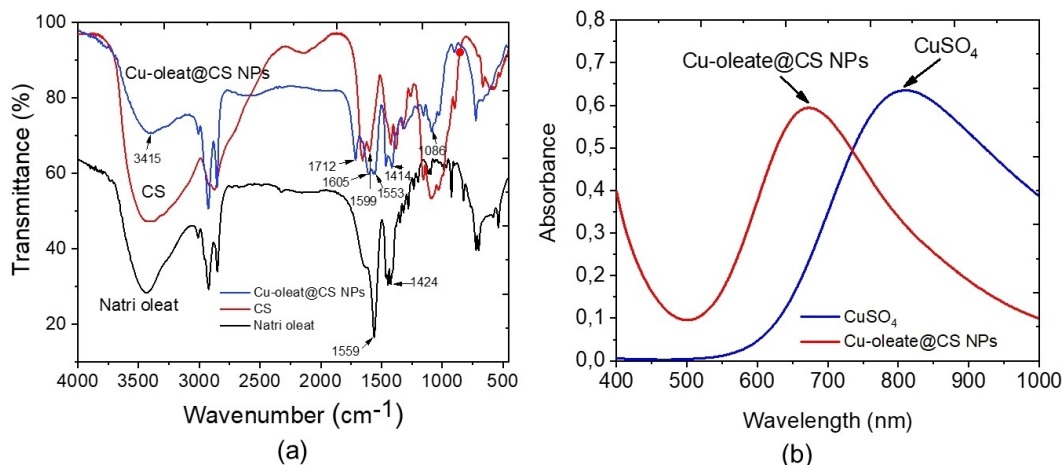


Figure 1. (a) FT-IR spectra of Cu-oleate@CS NPs; chitosan (CS); sodium oleate and (b) UV-Vis absorption spectra of Cu-oleate@CS NPs; CuSO₄.

has shifted to 1553 cm⁻¹ in the product, demonstrating the interaction of chitosan with copper oleate.

The above IR spectrum data proves that the copper oleate product was formed and coated with chitosan by the *in-situ* method. This conclusion is also clarified through the UV-Vis molecular absorption spectrum. The maximum absorption peak has a blue shift from the wavelength of 810 nm for the initial blue copper salt solution to 670 nm for the turquoise copper oleate nano solution (Fig. 1b), due to the interaction of the oleate group with Cu²⁺ ions.^[43]

The particle size of Cu-oleate@CS NPs was determined using ImageJ software and average particle size was calculated using Excel software. As the results, Cu-oleate@CS NPs were found to contain Cu with a content ranging from 5.0 to 6.0% by weight, of which the nanoparticles had an average particle size ranging from 50 nm to 60 nm, covered by a polymer layer with a thickness ranging from 10 to 12 nm (Figures 2a–d). Figure 2 presents a TEM microstructural morphology image of a Cu-oleate@CS NP suspension taken on a JEOL 2100 transmission electron microscope at magnifications of 10,000 times, 30,000 times, 50,000 times, and 200,000 times, respectively. The Cu-

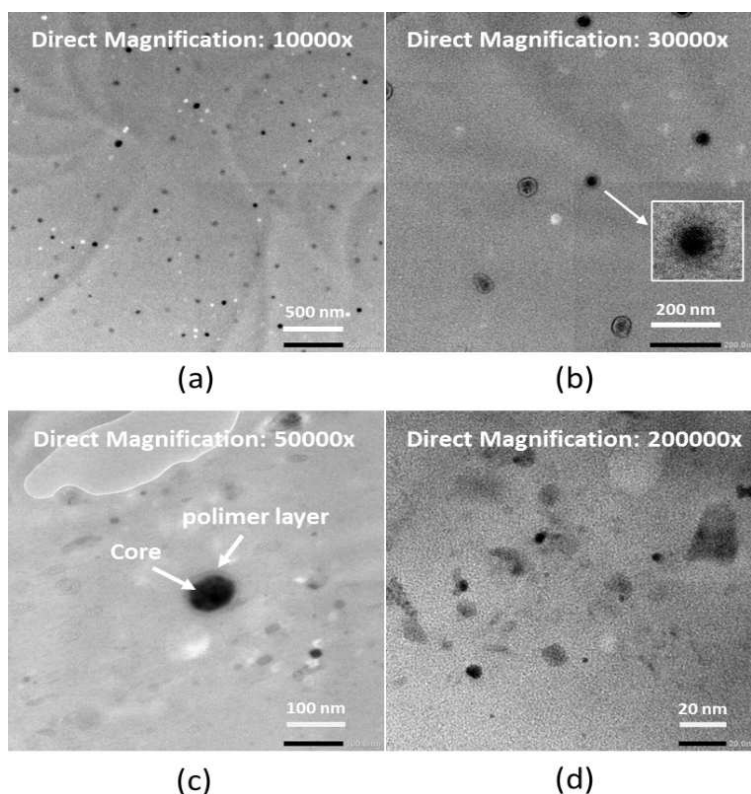


Figure 2. The typical TEM images of Cu-oleate@CS nanoparticles from different direct magnification

oleate@CS NPs produced by this method are uniform in size, spherical in shape, and have an average size ranging from 50 to 60 nm. Figure 2d is a TEM image of Cu-oleate@CS NPs with a magnification of 200,000 times, showing a sample with a small number of individual particles with small sizes, corresponding to 17 nm. The nanoparticle sizes obtained by TEM were smaller than those observed from DLS analysis, which is completely consistent, because the results of DLS analysis are dynamic in size, obtained from a very large collection of particles, while TEM images only observe a small area. The number of observed particles is small, the size obtained is the static size. On the other hand, the particle size of coated samples measured in a liquid phase (DLS measurement) is larger than when taking TEM images, which can be explained because when measuring particles in a liquid phase, hydrophilic polymers will be relatively large, interacts with water and makes the particles larger.

The elemental composition of Cu-oleate@CS NPs was determined by Energy-Dispersive X-ray spectroscopy (EDX) (Figure 3). The EDX results showed that the organic copper NPs contain Cu of 5.39%, C of 72.36%, O of 13.81%, and N of 8.44% by weight. In combination with the EDX spectra in Fig. 3a, the corresponding elemental mappings of Cu, C, N, and O elements are shown in the inset of Fig. 3b. The elements' map indicated that Cu, C, N, and O elements were evenly distributed in the Cu-oleate@CS NPs.

Stability of the Cu-Oleate@CS NPs

DLS results of Cu-oleate@CS NPs gave a single peak (corresponding to a particle size of 130 nm) as shown in Figure 4a, with absolutely no clumping, creating particle complexes in the size region. Particle size was mainly in the range of 90 to 200 nm. This confirmed that the nanoparticles are evenly dispersed in the liquid phase.

In aqueous phase, zeta potential is often used as a threshold to evaluate stability. The zeta potential represents the degree of repulsion between similarly charged particles close to each other in a dispersed system. For molecules and particles that are small enough, a high zeta potential (negative or positive) will provide high stability, and the dispersed system will resist flocculation and clumping. The measured zeta potential of the Cu-oleate@CS NP sample reached -61.7 mV (Figure 4b). Thus, the achieved zeta potential was quite optimal and confirmed the good stability of the Cu-oleate@CS NPs.

Characterization of Organocopper Nanoformulation Containing Cu-Oleate@CS NPs

To test the bioefficacy of the Cu-oleate@CS NPs, we formulated several organocopper nano-formulations that were suitable to spray and interact with plant surfaces. During the preparation of the nanoformulations, the organocopper nano-formulation containing Cu-oleate@CS NPs has a phase separation phenomenon in an aqueous solution due to the presence of a hydrophobic part (fatty acids). Therefore, in this research, we conducted a trial mixing in different recipes for three

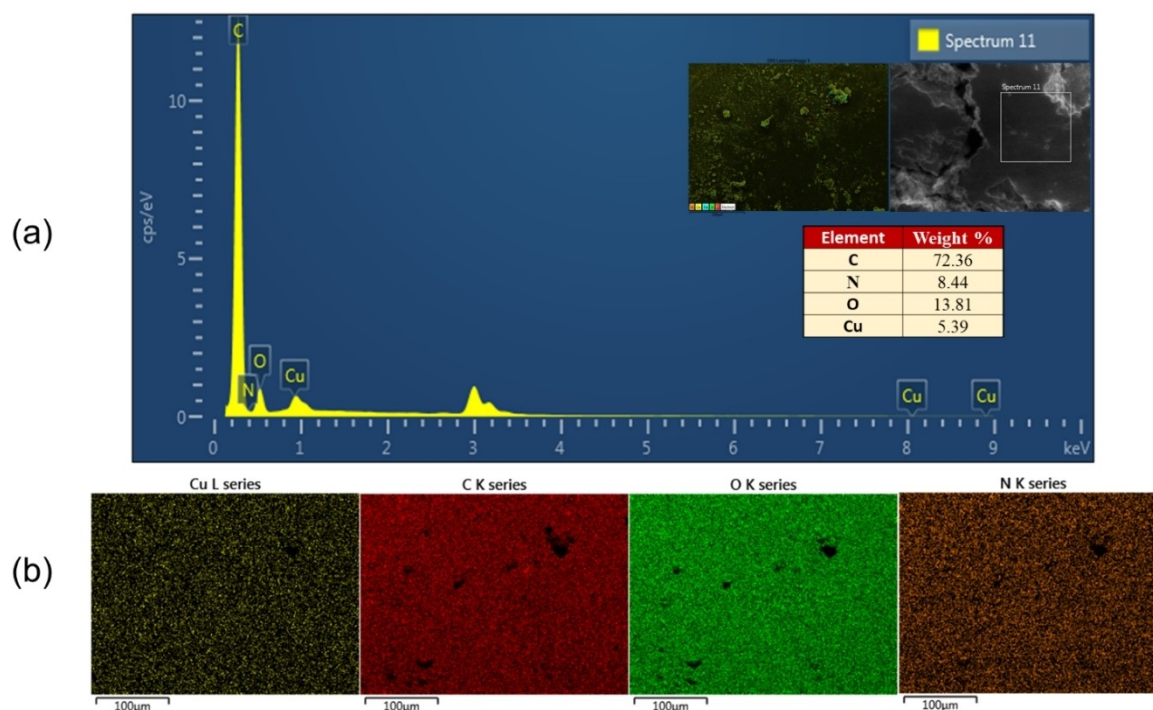


Figure 3. EDX images of Cu-oleate@CS NPs. (a) EDX spectrum and the percentages of elements in Cu-oleate@CS NPs and (b) EDX elemental mapping of Cu-oleate@CS NPs, with the inset showing by color the mappings of the four detected elements for the box in (a) image.

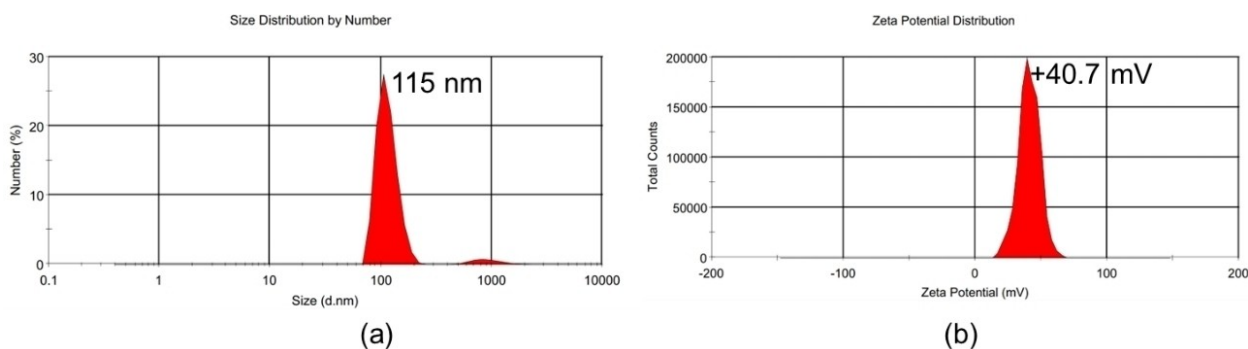


Figure 4. Dynamic light scattering (DLS) analysis of Cu-oleate@CS NPs. (a) Size distribution. (b) Zeta potentials of Cu-oleate@CS NPs. The size distribution and Zeta potentials of Cu-oleate@CS NPs were measured at a 2000-fold dilution.

formulations FCu1-3 to find one with the most suitable durability. Each formulation was prepared at a scale of 100 mL with a Cu-oleate@CS NP concentration of 2000 $\mu\text{g}/\text{mL}$ (Table 1).

The formulations were mixed and stirred for 60 min at 600 rpm and at 40 °C. The Cu-oleate@CS NP concentration was 2000 $\mu\text{g}/\text{mL}$ in all of FCu1-3.

The Cu concentration in each organocopper nanoformulation was re-determined by high-frequency inductive plasma source spectroscopy connecting with ICP-MS spectrometry. ICP-MS analyses of FCu1-3 showed values of Cu contents of 987.23, 962.51 and 954.63 mg/mL, respectively (data not shown). In addition, distributing particle size and the zeta potential stability of Cu-oleate@CS NPs in the organocopper nanoformulations were re-determined by dynamic laser scattering (DLS). In addition, the obtained organocopper nanoformulations FCu1-3 were evaluated for their stability by water solubility test at the ratio of 1 mL/100 mL water, 2 mL/100 mL water, and observed any changes in physical state as well as stability of the mixtures after 1 hour. (Figure 5).

In this research, we used the non-ionic surfactant Tween 80 (Polysorbate 80). With the solvent power in water, ethanol, and HLB value (Hydrophilic - Lipophilic Balance) = 15.0 will act as a solvent (solubilizer), stabilizer, and emulsifier (surfactant). As a result, the mixtures of liquid and oil-based components in the formulation containing Cu-oleate@CS NPs were easily mixed and had high stability compared with the mixture without any non-ionic surfactants.

Table 1. Ingredients of organocopper nano-formulation containing Cu-oleate@CS NPs.

Ingredient	Organocopper nano-formulation		
	FCu1	FCu 2	FCu 3
Cu-oleate@CS NPs	50 mL	50 mL	50 mL
Tween 80	10 mL	5 mL	0 mL
Axetone	5 mL	10 mL	15 mL
Ethanol	5 mL	10 mL	15 mL
Water	30 mL	25 mL	20 mL
Total	100 mL	100 mL	100 mL

The size distributions (DLS) and zeta potential stabilities of FCu1-3 are presented in Figure 6. The results show that the water polar solvent is proportional to the tween surfactant used. The hydrodynamic size distribution (DLS) of FCus gradually decreases in the order of FCu3, FCu1, and FCu2, corresponding to average sizes of 392 nm, 272 nm, and 75 nm, respectively. When adding water solvent, the amount of tween 80 decreases and the solution durability will decrease. Specifically, the zeta potential strength of Fs is +16.2 mV, +40.7 mV and +59.4 mV equivalent to FCu3, FCu1, and FCu2, respectively. To reduce the amount of surfactant, ethanol, and acetone were used to increase the dispersion of organic copper active ingredients. To reduce the cost of the formulation, the appropriate ratio of acetone/ethanol/water was selected as 1:1:2.5, in which Tween 80 accounted for 5%. The organocopper nanoformulation FCu2 presented a suitable large-scale production of finished products.

In our work, the natural polymer chitosan was used as a carrier, stabilizer, and coating for organic copper nanoparticles. Comparing the approach of synthesis of Cu-oleate@CS NPs to earlier reports on the synthesis of nanoparticles and the ex-situ encapsulation methods, our work presented a novel development. The drawback of the existing ex-situ encapsulation method for nanoparticle encapsulation is that the nanoparticles are coated with polymers like sodium alginate, chitosan, and polyethylene glycol after they are created. This led to the stage that the particles have low monodispersity and poor solubility in water because they are easily clustered and large in size due to not being microencapsulated during the nanocrystal creation process. As a result of the method used in our work, Cu-oleate@CS NPs release and dispersion may be better managed. Additionally, the Cu-oleate@CS NPs was better prevented from excessively rapid degradation, leading to prolongation of their antifungal activity.

In Vitro Cytotoxicity Results

The toxicity of the Cu-oleate@CS NPs was tested with the Vero cell line according to the SRB method,^[44] the results are shown in Figure 6.

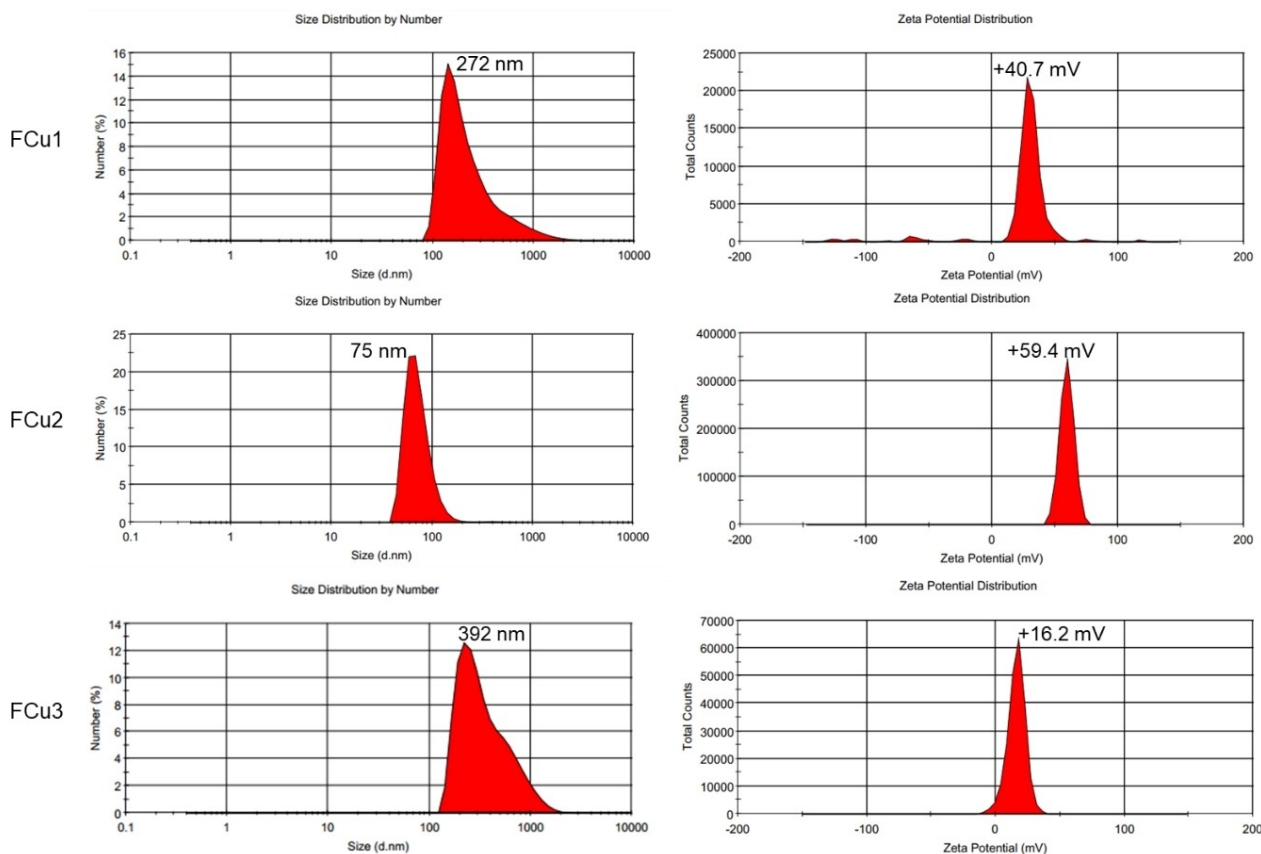


Figure 5. The size distributions and zeta potentials of Fcu1-3 organocopper nanoformulation containing Cu-oleate@CS NPs. The size distribution and Zeta potentials of organocopper nanoformulations were measured at a 2000-fold dilution.

The results showed that DMSO with the tested concentrations had no cytotoxic effect on 100% proliferative cell. In contrast, the standard substance in the positive control sample had a toxic effect, killing most healthy Vero cells. For the tests with Cu-oleate@CS NPs, the proliferative cell rate on the Vero cell line in equivalent with 98.65%, 97.78%, 87.64%, 68.37% and 41.79% correspond to the survey concentrations of 5 $\mu\text{g}/\text{mL}$; 10 $\mu\text{g}/\text{mL}$, 25 $\mu\text{g}/\text{mL}$; 50 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$, respectively. The morphology of Vero cells observed using an inverted microscope is presented in Figure 6a. This figure shows that the morphology of the cells was significantly affected and unchanged by the presence of Cu-oleate@CS NPs at a concentration range of 5 to 50 $\mu\text{g}/\text{mL}$. Only at concentrations as high as 100 there were begin to be an effect on cell survival and morphology with rates exceeding 50% of death cell. Hence, it showed that the Cu-oleate@CS NPs nano system has a low toxic effect on the healthy Vero cell line. The low toxicity of our Cu-oleate@CS NPs was possible due to the high percentage of oleate in the nanoparticles and the fraction of Cu^{2+} released ions in lower concentrations than nanoparticles containing only Cu or Cu_2O and to the coverage of the biocompatible polymer chitosan.

In Vitro Inhibitory Activity of Cu-Oleate@CS NPs Against the Mycelial Growth of *Colletotrichum Gloeosporioides*

The fungal inhibitory bioassay (food-poisoned technique) was employed to evaluate the antifungal effects of Cu-oleate@CS NPs on mycelial growth of *C. gloeosporioides* (C-G), a well-known pathogenic agent of anthracnose in oranges.

Cu-oleate@CS NPs were suspended in water with Tween 80 (0.05%) and DMSO 2% to form suspensions at a concentration of 500 $\mu\text{g}/\text{mL}$ (equivalent to a Cu content of 28 $\mu\text{g}/\text{mL}$) and 1000 $\mu\text{g}/\text{mL}$ (equivalent to Cu content of 56 $\mu\text{g}/\text{mL}$).

As illustrated in Table 2, Cu-oleate@CS NPs inhibited *C. gloeosporioides* by 51.5 to 75.7% at both tested concentrations of 500 and 1000 $\mu\text{g}/\text{mL}$ (Table 2). Cu-oleate@CS NPs showed high antifungal activity, at 500 $\mu\text{g}/\text{mL}$; it caused an inhibition of 59.4% of *C. gloeosporioides* 3 days after treatment. The antifungal effectiveness of Cu-oleate@CS NPs still reached 51.5% at 5 days after treatment. Particularly, Cu-oleate@CS NPs produced a remarkable inhibitory value of 75.7% against the mycelial growth of *C. gloeosporioides* 3 days after treatment at 1000 $\mu\text{g}/\text{mL}$ (equivalent to 56 $\mu\text{g}/\text{mL}$ of Cu content) and continued to reach above 70% after 5 days (Figure 7). According to Kozlevcar et al. (2000), copper (II) carboxylates $[\text{Cu}_2(\text{O}_2\text{CC}_n\text{H}_{2n+1})_4(\text{urea})]$ ($n=5$ to 11) with urea were active against wood decay fungi *Trametes versicolor* and *Antrodia vaillantii* at concentrations of

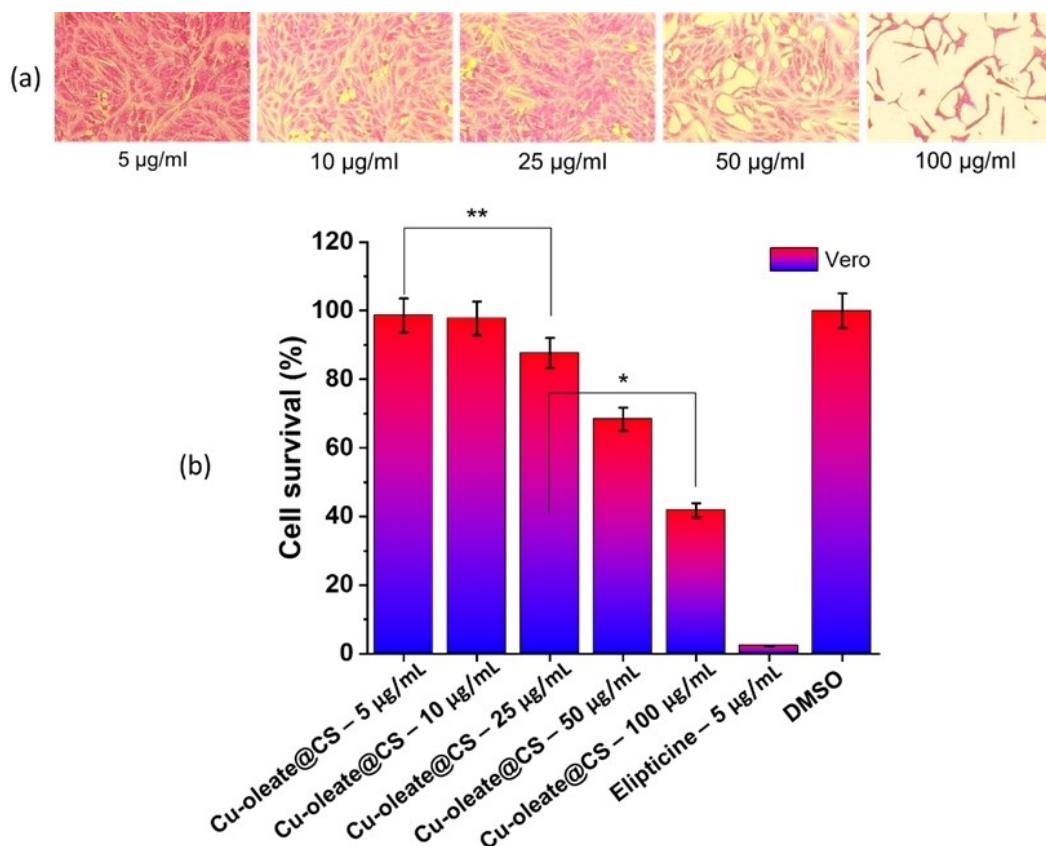


Figure 6. Cytotoxicity of Cu-oleate@CS on Vero. (a) Vero cell treated with a range of concentration of Cu-oleate@CS. Microscope images were taken with objective lens: 10X, and (b) Percentage of cell survive of samples incubated with Cu-oleate@CS NPs (5-10-25-50–100 µg/mL); DMSO and Ellipticine (5 µg/mL).

Table 2. *In vitro* inhibitory activity of Cu-oleate@CS NPs against the mycelial growth of *Colletotrichum gloeosporioides* (C–G) after 3 and 5 days.

Sample	Conc. (mg/mL)	3 Days		5 Days	
		Mycelial Diameter (mm)	Inhibitory efficacy (%)	Mycelial Diameter (mm)	Inhibitory efficacy (%)
Cu-oleat@CS NPs	0.5	12.4 ± 0.50 ^b	59.4 ± 1.70 ^b	22.4 ± 0.90 ^b	51.5 ± 1.50 ^b
	1	9.0 ± 0.35 ^c	75.7 ± 2.38 ^a	15 ± 0.76 ^c	71 ± 2.25 ^a
Control		24.6 ± 0.96 ^a		41.9 ± 1.43 ^a	

Cu-oleate@CS NPs were mixed into molten sterilized PDA medium at 50 °C, and then cooled to the solidity of PDA before inoculating *C. gloeosporioides* and incubating at 25 °C for 3–5 days. Control was treated with water alone. The data presented are means of three replicates with standard deviation. Data followed by the same letters in the same column are not significantly different at $P \leq 0.05$ level according to the Duncan's multiple range test.

10^{-4} to 10^{-3} mol/L.^[43] Copper (II) soaps at concentration ranging from 1 to 10 mg/mL were also reported to moderately inhibit the mycelial growth of *Alternaria alternata* and *Aspergillus niger* *in vitro*.^[15] The previous *in vitro* data in partly supported our findings of antifungal potent of copper (II) oleate. However, our study was the first investigation of the antifungal activity of Cu-oleate@CS NPs against *C. gloeosporioides*. The *in vitro* antifungal evidence of Cu-oleate@CS NPs resulted in hypothesis that they might have an impact on citrus anthranoses, which in turn prompted a field trial of the nanoparticles on orange plants. (Figure 8).

Field Trial of Organocopper Nanoformulation on Orange Plants

Testing results to monitor disease index and calculate biological effectiveness of organocopper nanoformulation FCu2 to control orange anthracnose on field trials, shown in Table 3. To spray FCu2 on orange plants, the doses of FCu2 were prepared at 0.25 (CT1), 0.33 (CT2), and 0.55% (CT3) in water and sprayed run-off on the test plant plots. Disease severity index (DSI) and Disease control efficacy (DCE) of organocopper nanoformulation to prevent orange anthracnose were assessed at 7 and 14 days after sprayings.

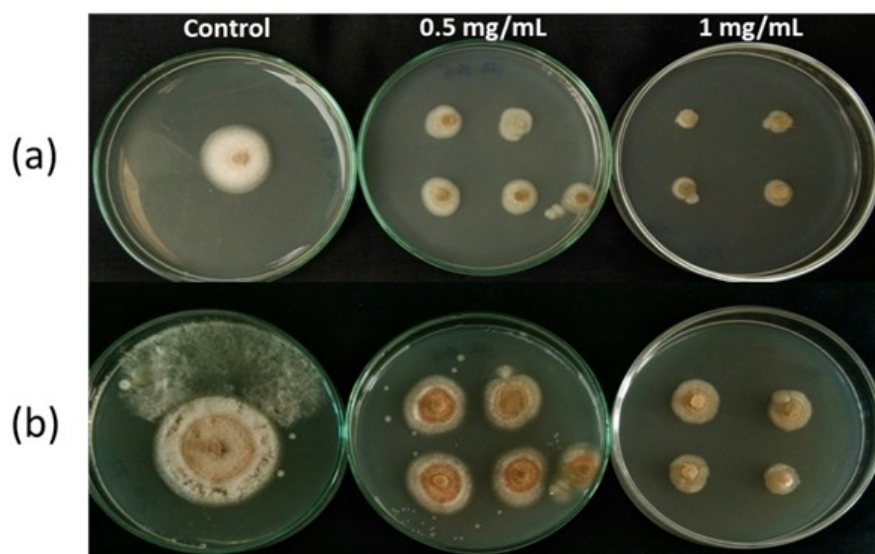


Figure 7. *In vitro* effects of Cu-oleate@CS NPs on the mycelial growth of *C. gloeosporioides* at 500 and 1000 $\mu\text{g}/\text{mL}$. The fungus was cultured in Petri dishes and incubated at 25 °C. The mycelial diameters were recorded after 3 (a) and 5 (b) days.

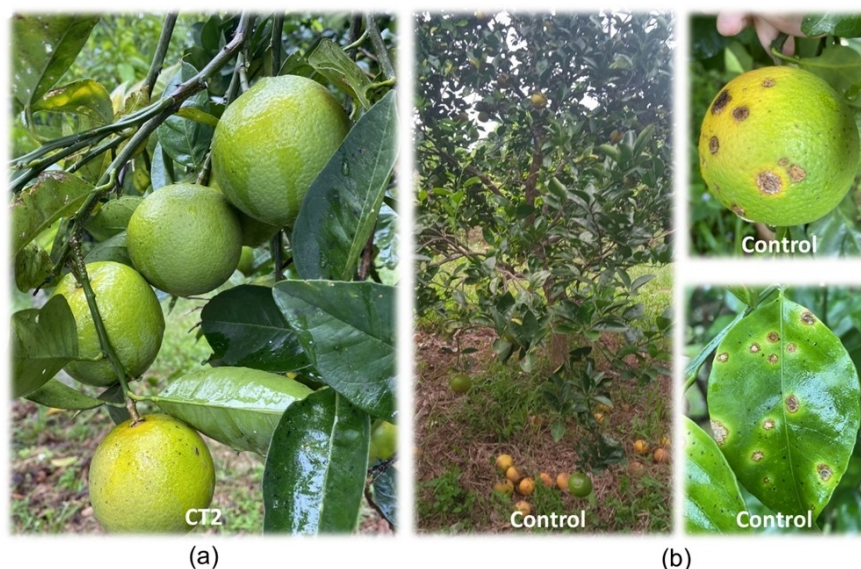


Figure 8. Citrus anthracnose symptoms in *Citrus sinensis* (type Van Du) treated with organocopper nanoformulations CT1-3. The symptoms were observed 14 days after the second application (a) and negative control (b). The field trials were conducted from September to November 2021 in Thanh Chuong districts of Nghean province in Vietnam.

In the field trials, the disease control efficacy of the organocopper nanoformulations against citrus anthracnose in orange plants at 7 and 14 days after treatment were assessed (the assessments of 7DA1T, 7DA2T, and 14DA2T) and shown in Table 3. Before the 1st treatment, the DSI values of all plots were 4.96 to 5.12% and there were no statistically significant differences between each investigated plot (Column 3 Table 3). However, the DSI increased at 7 days of post-treatment. Of these, CT2 caused the lowest DSI value of 7.16% while CT1, CT3, and control showed CSI values higher than 9.05%. All DSI values of CT1, CT3, and control were not statistically different. It

implied that CT2 was the best active formulation at 7 days post-treatment.

The assessment at 7 days after the 1st spraying demonstrated that the nanoformulation CT2 at a dose of 0.33% suppressed by 57.27% DCE for citrus anthracnose; it proved to be more effective than formulations CT1, CT3, which only reached DCE values of 28.45% and 45.92%, respectively.

The highest DCE values of CT1-3 were found to be 45.05–64.59% at 7 days after the 2nd spraying. Interestingly, all nanoformulation CT1-3 still controlled anthracnose in orange with DSI from 6.95 to 10.78%, which was significantly lower than that of control (19.72%) in the 2nd assessment. At 7 days

Table 3. Disease incidence (%) anthracnose and disease control efficacy of organocopper nanoformulation in plants 7, 14 days after applications in field trials from September to November 2022.

Formula	Conc. (%)	Disease severity incidence and disease control efficacy (%)						
		Before treatment	7DA1T		7DA2T		14DA2T	
		DSI (%)	DSI (%)	DCE	DSI (%)	DCE (%)	DSI (%)	DCE (%)
CT1	0.25	4.96 ± 0.24 ^a	12.06 ± 0.50 ^a	28.45 ± 0.68 ^c	10.78 ± 0.52 ^b	45.05 ± 0.78 ^a	12.69 ± 0.60 ^b	39.12 ± 0.90 ^b
CT2	0.33	5.03 ± 0.32 ^a	7.16 ± 0.35 ^b	57.27 ± 0.70 ^b	6.95 ± 0.30 ^{bc}	64.59 ± 0.52 ^b	9.36 ± 0.48 ^b	54.18 ± 0.56 ^{bc}
CT3	0.50	4.98 ± 0.30 ^a	9.05 ± 0.42 ^b	45.92 ± 0.58 ^a	8.37 ± 0.45 ^b	57.26 ± 0.80 ^a	11.05 ± 0.76 ^b	48.32 ± 0.76 ^a
Control	0	5.12 ± 0.27	17.12 ± 0.67 ^a	–	19.72 ± 0.65 ^a	–	21.46 ± 0.85 ^a	–
LSD _{5%}				25.56		15.27		12.39
CV%				5.37		6.12		5.73

Note: TPL1: Before the 1st treatment; 7DA1T: 7 days after the first treatment; 7DA2T: 7 days after the second treatment; 14DA2T: 14 days after the second treatment; DSI: Disease Severity Index (%); DCE: Disease Control Efficacy (%); The data presented are means of three replicates with standard deviation. Data followed by the same letters in the same column are not significantly different at $P \leq 0.05$ level according to the Duncan's multiple range test.

after the 2nd spraying, CT2 caused the best suppression against anthracnose in orange with a DCE of 64.59%. At 14 days after the 2nd spraying, the DCE value of CT2 was found to decrease to 54.18%. In contrast, DCE values of CT1 and CT3 only reduced by 39.12% and 48.32% respectively. CT1 caused the lowest DCE value against anthracnose in the 3rd assessment. The hypothesized cause for the unexpected result could be that the concentration of Cu nanoparticles in CT1 was too low.

The side effects of the organocopper nanoformulations CT1–3 on the field environment and beneficial organisms were checked during the field trials. The phytotoxicity testing process was performed according to the guidance of Vietnam MARD.^[45] The adverse effects of test nanoformulations such as the impact of the test samples on the environment and beneficial organisms will be recognized if it has. In fact, organocopper nanoformulations were observed to be safe for the environment and easily decompose within a certain period. The phytotoxicity symptoms on oranges in the field trials were found to occur rarely. The development and growth of orange plants were not significantly impacted by the organocopper nanoformulations CT1–3. Even though applying the highest dose of CT3, the treated orange plants only displayed mildly yellow leaves and light symptoms of phytotoxicity. However, the plants immediately recovered during our assessments (Figure S1). In the future, larger field trial models should be conducted to verify in more detail the DCE of CT2 nanoformulation and its side effects on plants during the different seasons and in other orange plantations.

Conclusions

For the first time, Cu-oleate@CS NPs were successfully fabricated by using natural chitosan polymer as a coating agent and stabilizer in an exchange reaction combined with *in-situ* coating method. The morphology, stability, and antifungal properties of the nanoparticles were determined. Cu-oleate@CS NPs were composed of Cu (5.68%), C (70.96%), O (13.32%), and N (7.69%), and the suspensions of Cu-oleate@CS NPs obtained

were in turquoise liquid form and had a Cu content ranging from 5.0 to 6.0% by weight. Cu-oleate@CS NPs possessed an average particle size ranging from 50 nm to 60 nm (TEM) and covered by a layer of polymer with a thickness of about 10 to 12 nm. The analyses of zeta potential stability and dynamic solution size of Cu-oleate@CS NPs in suspensions showed that the nanoparticles are uniformly dispersed (90–120 nm by DLS), with high stability (zeta potential of –61.7 mV) and low toxicity Vero healthy cell line at high test concentrations. Cu-oleate@CS NPs were significantly active against *C. gloeosporioides in vitro* at 500 and 1000 µg/mL, equivalent to 28 and 56 µg/mL of Cu contents. Moreover, organocopper nanoformulation CT2 exhibited the best suppression (64.59% DCE) against citrus anthracnose in oranges was observed at a dose of 0.33%, and its DCE was still maintained at 54.18% at 14 days after the second spraying. Our study results of Cu-oleate@CS NP fabrication and its organocopper nanoformulation suggested a new and green material that could be effectively used for controlling the citrus anthracnose in orange plants *in vivo*.

Supporting Information Summary

The Supporting Information includes the detailed experimental section of this work

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Conflict of Interests

The authors declare no conflict of interest

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Keywords: Pesticides · organocopper nanoparticles · bio efficacy field trials · citrus and anthracnose

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