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Fungicidal activities of chitosan-stabilized copper nanoparticles on *Magnaporthe oryzae*, *Rhizoctonia solani*, and *Phytophthora capsica*

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Abstract

Pathogenic fungi are the most common causes of economic loss in crop production. Until now, synthetic fungicides are the most effective tools for management in agriculture, but they cause a severe impact on the environment as well as several side effects for human health. The use of synthetic fungicides is prohibited in organic agriculture; however, sulfur and copper fungicides are still permitted in organic farming. In this study, chitosan-stabilized copper chitosan nanoparticles (CS-CuNPs) were synthesized and characterized via UV-Vis analysis and scanning electron microscopy (SEM). Moreover, fungicidal activities of CS-CuNPs in the range of concentrations (00, 500, and 1000 mg/L) against several plant pathogenic fungi, including *Magnaporthe oryzae*, *Rhizoctonia solani*, and *Phytophthora capsici*, have been assessed via filamentous fugal growth inhibition. The results indicated that the size of CS-CuNPs ranged from 70 to 74 nm with the plasmon absorption peak at 600 nm, which implied the CS-CuNPs were successfully synthesized. Furthermore, CS-CuNPs effectively inhibited the growth of all three fungi at a concentration 1000 mg/L. Among the three species, *Rhizoctonia solani* was the most susceptible to CS-CuNPs, with the growth inhibitory effect at 100 mg/L. In conclusion, CS-CuNPs demonstrated a strong potential for the elimination of plant pathogenic fungi and further applications in agriculture.

Introduction

Several fungal plant diseases, such as blast disease, sheath blight, and foot rot disease, might be a significant threat to crop production and food safety (Skamnioti and Gurr 2009). Rice blast, which is caused by the filamentous fungus *Magnaporthe oryzae*, is one of the most destructive cereal diseases and results in losses of 10 – 30 % of the global yield of rice (Nalley *et al.* 2016). It is undeniable that rice is the most important crop, feeding more than half of the human population and contributing about 19 % of the daily calories consumed across continents (Elert 2014). In addition, *M. oryzae pv. Triticum* also causes wheat blast, an emerging threat to wheat cultivation around the world, especially in South America and Asia (Kohli *et al.* 2011; Portz *et al.* 2020). *Rhizoctonia solani* is the pathogen that causes rice sheath blight, one of the most serious fungal diseases of rice, resulting in significant yield losses in many rice-growing regions of the world

(Matsumoto and Cuong 2014). This pathogen significantly affects not only the yield and quality of rice when harvested but also other plants such as corn, barley, soybeans, sugarcane, and strawberries (Schurt *et al.* 2014). *Phytophthora capcisi* is identified as the cause of rapid death of black pepper and is the main pathogen causing Phytophthora foot rot disease of black pepper in Vietnam (Truong *et al.* 2008). *Phytophthora capcisi* infects and attacks pneumatophores and underground parts. When roots and stems are damaged, the pepper plant will suddenly die. Root rot as well as damage to the phloem vessels and plant tissues restrict the transport of water and nutrients from the roots to the upper parts of the plant (Ravindran *et al.* 2000). That may cause the rapid death of black pepper and reduce its yield. Currently, synthetic fungicides remain the major methods to control plant pathogenic fungi in agriculture, but they cause environmental pollution, soil degeneration, harmful human health effects, and poor agricultural product quality with high residues of pesticides. In addition, chemicals have disadvantages against several phytopathogenic fungi that are fungicide resistant pathogens and have restricted use in organic farming. Biological agents are safer for the environment and human health; however, they are less effective than synthetic fungicides and have a slow effect on the control of plant diseases (Chandrashekara *et al.* 2012). Therefore, the development and application of new fungicides that have high effectiveness and less adverse effects on the environment and human health are in high demand for crop protection. According to Mustafa *et al.* (2018), sulfur and copper-based fungicides could meet the requirements of organic farming, and the copper nanoparticle fungicide is less toxic as compared to the conventional copper-based fungicide (Adwecopper). That suggests a promising approach of nanotechnology in the control of plant diseases in agriculture.

Nanotechnology is an emerging technology for the development and production of advanced structures and materials at the nanoscopic scale and has a broad range of applications in various sectors of human life, such as science, engineering, industry, food preservation, medicine, and agriculture. According to Fraceto *et al.* (2016), nanotechnology not only provides materials to enhance soil quality and plant growth but can also be used to develop new sensor materials for smart monitoring systems or new formulations of nanofertilizers and nanopesticides to improve crop production and food security. Of note, the protection effects of some metal nanoparticle types against plant diseases, including fungal diseases, have been well documented in the literature (Kutawa *et al.* 2021). Metal nanoparticles could be produced via bottom-up (such as solid-state methods, liquid-state synthesis methods, gas phase methods, biological methods, and others) or top-down (including mechanical milling, laser ablation, and sputtering) approaches (Jamkhande *et al.* 2019). Depending on their size, shape, and synthesis techniques, metal nanoparticles have different efficacy and mechanisms of action against microbes. Mechanisms of anti-fungal activity of metal nanoparticles range from membrane damage, damage to hyphae and spores, alteration of biofilm formation, mitochondrial dysfunction, ion release, ROS generation, DNA interactions, change of gene regulation and protein levels (Slavin and Bach 2022). Among them, copper nanoparticles (CuNPs) have a promising potential for application in agriculture because they are more effective tools to control the wide-ranging plant pathogenic fungi, and copper-based fungicides can be used in organic farming, the agricultural practice and system for healthier food production and the eco-friendlier as well as sustainable approach to agriculture development. (Mustafa *et al.* 2018; Ibarra-Laclette *et al.* 2022). For example, the inhibitory effect of green-synthesized CuNPs on mycelial growth of *Fusarium solani*, *Neofusicoccum* sp., and *Fusarium oxysporum* has been reported (Pariona *et al.* 2019). Moreover, Brunel *et al.* (2013) have suggested the synergistic effect of the complexion of copper and chitosan nanogels to impede *Fusarium graminearum* growth, which proposes a new approach to developing chitosan-stabilized copper nanoparticles (CS-CuNPs) for crop protection.

However, the fungicidal effects of CS-CuNPs against *M. oryzae* and *R. solani*, which cause major diseases in rice, one of the main crops in Vietnam, such as rice blast and rice sheath blight, and *P. capsica*, which causes the foot rot disease in black pepper in Vietnam, have not been elucidated yet. In this study, we investigate the synthesis process of CS-CuNPs and its antifungal effect against a variety of major plant pathogenic fungi, including *M. oryzae, R. solani*, and *P. capsici*, to test the potential use of CS-CuNPs as fungicides.

Experimental

Copper chitosan nanoparticle (CS-CuNPs) synthesis

CS-CuNPs were synthesized by using chitosan as a protector, and ascorbic acid and $NaBH_4$ (Sigma-Aldrich) as an ion copper de-salters to form the nanoparticles. 4 g of Chitosan were first dissolved in 4 mL of lactic acid. 100 mL of distilled water were supplemented. After that, a volume of $CuSO_4$ solution (0.8 g $CuSO_4 \cdot 5H_2O$ dissolved in 20 mL of distilled water) and a volume of ascorbic acid solution (0.88 g ascorbic acid dissolved in 20 mL distilled water) was gradually added. Finally, $NaBH_4$ solution (0.296 g $NaBH_4$ dissolved in distilled water) was added to the mixture. The resulting mixture was stirred with a speed of 450 – 500 rpm at room temperature for 2 h until the solution turned red to complete the preparation of the CS-CuNPs solution. All chemicals were from Sigma-Aldrich (Saint-Louis, USA).

UV-Vis analysis

The absorption spectrum of new synthesized CS-CuNPs was determined using an UV-Vis spectrophotometer (Hitachi U-2900, Hitachi High-Technologies Cooperation, Tokyo, Japan) to examine the availability of nanoparticles. The existence of CuNPs was confirmed by peak formation between 570 – 600 nm.

Characterization of CS-CuNPs.

The particle size, state, and shape of CS-CuNPs in solution were analyzed via scanning electron microscopy (SEM) (Hitachi F4800, Hitachi High-Technologies Cooperation, Tokyo, Japan). Specific particle size was determined using Image J software version 1.52v from SEM images of the samples.

Determination of the in vitro antifungal effect of CS-CuNPs

Fungal isolates of *M. oryzae, R. solani*, and *P. capsici* were provided by the Biotechnology Laboratory of the Research and Development Center (Saigon Hi-Tech Park, Vietnam). CS-CuNPs at concentrations of 100, 500, and 1000 $mg.L^{-1}$ were supplemented with potato dextrose agar (PDA) plates. A biomass culture of either *M. oryzae, R. solani*, or *P. capsici* was placed at the center of agar plate and incubated at 37 °C. The fungus cultured on a plate without nanoparticles served as a negative control. The growth inhibition percentage (GI %) was calculated according to the following formula (Eq. 1):

$$GI\ (\%) = [(D-d)/D] \times 100 \qquad (1)$$

where D is radial growth of fungal mycelia on the negative control plate when the fungus was fully grown (cm); d is the radial growth of the experimental plates (cm).

Statistical analysis

The data were shown as the Mean ± SEM (standard error of mean) from triplicate experiments, the differences among groups were analysed via ANOVA One Way and Fisher's Least Significant of Difference test with statistical significance set at 0.05.

Results

Preparation of CS-CuNPs.

The absorption spectrum of the sample measured right after CS-CuNPs synthesis showed an absorption peak at 600 nm wavelength (Fig. 1). Several studies suggest that CuNPs have a maximum absorption peak around 550 – 600 nm and that phenomenon can be used as an indicator for CuNPs existence in solution (Creighton and Eadon 1991; Dhas *et al.* 1998). Furthermore, the newly synthesized CS-CuNPs were further characterized by SEM image (Fig. 2). As shown in Fig. 2, CS-CuNPs were spherical or elliptical nanoparticles with sizes ranging from 70 to 74 nm. Taken together, the data indicated the CS-CuNPs were successfully synthesized.

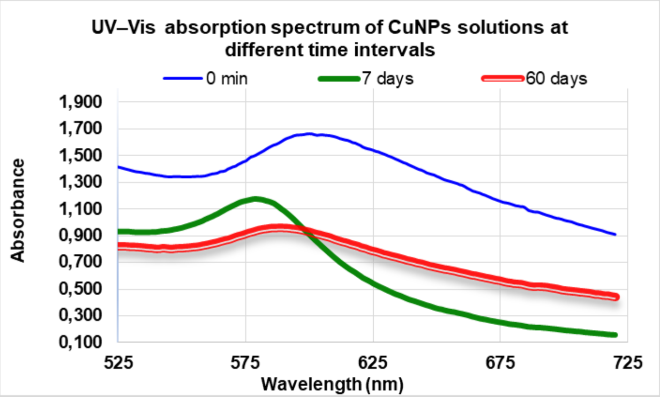

Fig. 1. UV-Visible absorption spectra of CS-CuNPs after 0, 7, and 60 days of fabrication.

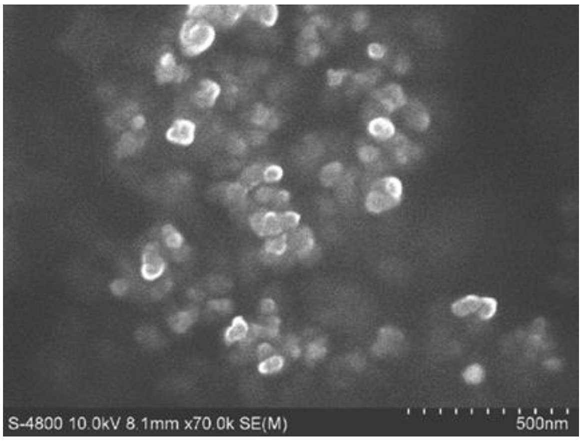

Fig. 2. SEM image of CS-CuNPs.

After 7 days, the absorption peak shifted to 579 nm, and the absorption density of CS-CuNPs decreased significantly. Until 60 days after CS-CuNPs fabrication, the absorption peak changed to 588 nm and the density declined as compared to days 0 and 7. The data indicated the transformation and aggregation of CS-CuNPs after fabrication, which led to a decline in the density absorption of CS-CuNPs. However, after 60 days of CS-CuNPs, the absorption peak was still in the reference range of copper nanoparticles (570 – 600 nm), which indicated the existence of CS-CuNPs. The data suggest that the CS-CuNPs aggregation and transformation occur with time, and further experiments and approaches need to be investigated to improve the stability of CS-CuNPs.

Inhibitory effect of CS-CuNPs on mycelial growth of M. oryzae, P. capsici, and R. solani

The fungicidal effects of CS-CuNPs were evaluated via the inhibition of mycelial growth radius in three pathogenic fungi, *M. oryzae*, *P. capsici*, and *R. solani* (Fig. 3 and Fig. 4). The color of the fungus growing in the control was brighter than that of the fungus treated with CS-CuNPs. The mycelium of treated samples turned into a darker color, showing aging and death (Fig. 3). The data revealed that CS-CuNPs were able to inhibit the growth of all three studied fungi. Moreover, the effectiveness of inhibition depended on fungal strains. For example, the mycelia of *M. oryzae* and *P. capsici* were partially degenerated at the concentration of 1,000 $mg.L^{-1}$ of CS-CuNPs, whereas those of *R. solani* were completely deteriorated (Fig. 3). That implied that *R. solani* was the most susceptible strain to CS-CuNPs, followed by *P. capsici* and *M. oryzae*. As shown in Fig. 4, the higher the concentration of CS-CuNPs used, the stronger the growth inhibition observed. Mycelial growth radius of *R. solani* was decreased to 40 % as compared to the control after treatment with 100 $mg.L^{-1}$ of CuNPs. Then it dramatically declined to 73 % and 89 % when the concentration of CS-CuNPs increased to 500 and 1,000 $mg.L^{-1}$, respectively. In *M. oryzae*, when treated with 1,000 $mg.L^{-1}$ of CS-CuNPs, the fungus was depressed, with an inhibitory percentage of about 64.22 %. However, at lower concentrations of CS-CuNPs 100 $mg.L^{-1}$ and 500 $mg.L^{-1}$, the inhibitory effect of the nanoparticles on the growth of *M. oryzae* was low at 14.64 % and 26.56 %, respectively. Furthermore, CS-CuNPs exhibited a strong fungicidal effect against *P. capsici*, ranging from 15.21 %, 40.49 %, and 65.54 % with a variety of concentrations of 100, 500, and 1,000 $mg.L^{-1}$, respectively. The results show that CS-CuNPs possess a strong fungicidal effect on three plant pathogenic fungi, and *R. solani* is the most susceptible strain to CS-CuNPs among the three species, which suggests the further application of CS-CuNP as a fungicide for plant pathogenic fungi management, especially in organic agriculture.

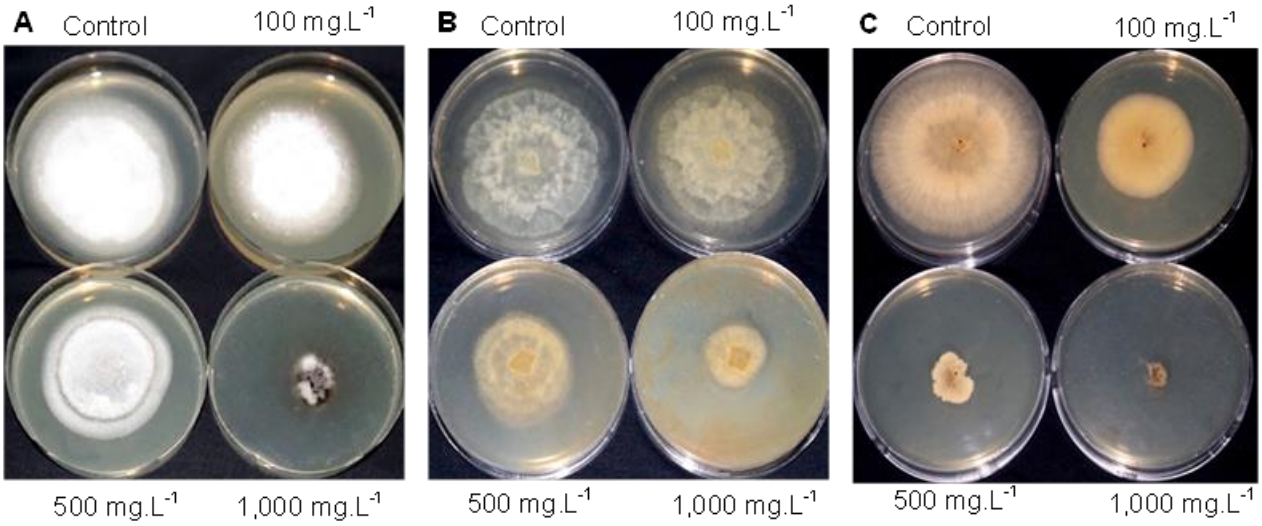

Fig. 3. Mycelial growth inhibition of A – *M. oryzae*, B – *P. capsici*, C – *R. solani* treated by different concentrations of CS-CuNPs (100, 500, and 1,000 mg.L^{-1} of CS-CuNPs).

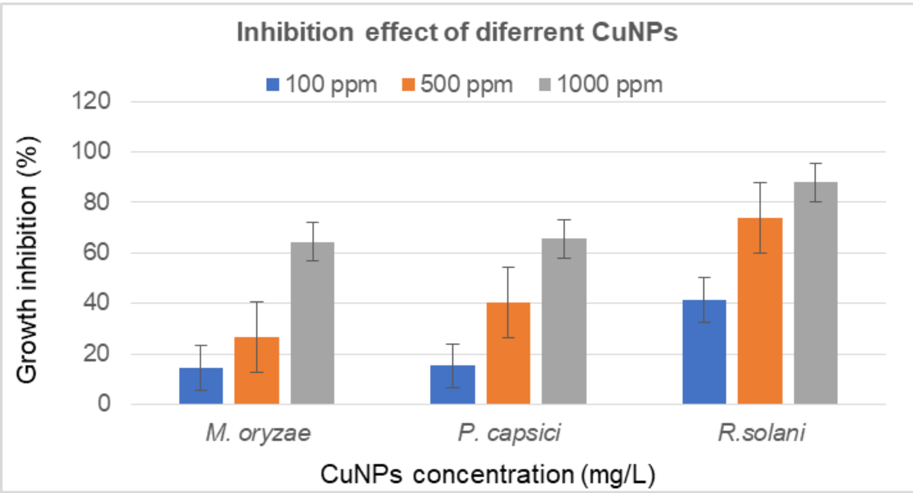

Fig. 4. Growth inhibition rate of *M. oryzae*, *P. capsici*, and *R. solani* when treating with different concentrations of CS-CuNPs.

Discussion

Different synthesis methods and conditions can produce a broad spectrum of metal nanoparticles sizes. For example, the sizes of CuNPs reported in previous studies vary widely, mainly due to different chemical reduction methods. According to Liu *et al.* (2012), the average size of the CuNPs reduced with increasing excess of $NaBH_4$ to copper ions, ranging from 42 to 115 nm. On the contrary, Al-Hakkani (2020) prepared CuNPs with a similar method and synthesized CuNPs with a size of about 30 – 100 nm. Furthermore, the different reducing and stabilizing agents may affect the nanoparticle size. In a previous study, CuNPs prepared by the reduction method using copper sulphate, borohydride, and trisodium citrate obtained smaller sizes ranging from 30 to 50 nm (Sagadevan and Koteeswari 2015). Viet *et al.* (2016) synthesized CuNPs via chemical reduction method with spherical nanoparticles ranging from 20 nm to 50 nm. In the present study, we used ascorbic acid and $NaBH_4$ as reducing agents and chitosan as a stabilizing agent and obtained spherical or elliptical nanoparticles, which were in the normal range of CuNPs. That indicates that the synthesis process has been successfully established and can be used to produce CS-Cu-NPs in the future. Furthermore, new synthesized CS-CuNPs are still in solution after 60 days, but they also aggregate and transform during storage, which suggests further investigation to prevent the loss of CS-Cu-NPs and enhance their stability.

Synthetic fungicides are the main tools to control plant pathogenic fungi and protect crop production. However, the overuse of synthetic fungicides could have harmful effects on human health and the environment and develop conventional fungicide resistance. A residue-free nanotechnology could be an effective strategy not only for its effectiveness on fugal growth inhibition but also for diminishing negative impact of fungicide residues on the environment (Xue *et al.* 2014). Note that, copper-based nanoparticles are also promising alternative materials to effectively control plant pathogenic fungi and deal with conventional fungicide resistance (Kanhed *et al.* 2014; Saharan *et al.* 2015; Malandrakis *et al.* 2022). For example, CuNPs exhibit a strong fungicidal effect against two plant pathogenic fungi, *Fusarium oxysprorum* and *Phytophthora capsici* (Pham *et al.* 2019). Moreover, copper oxide nanoparticles could also effectively control rust disease in coffee, and copper is one of the eight essential micronutrients in plants, which suggests the beneficial effect of nanoparticles on the plant (Leal *et al.* 2023). Recently, a new nanoparticle, named chitosan-stabilized copper nanoparticles was synthesized and showed an anti-cancer effect against bladder cancer (Hongfeng *et al.* 2021). In this study, CS-CuNPs exhibit anti-fungal activity against all tested microorganisms, such as *M. oryzae*, *P. capsici*, and *R. solani*, which implies the potential application of CS-CuNPs as a new tool for controlling plant pathogenic fungi. Among the three fungi, *R. solani* is more sensitive to CS-CuNPs than the others, which suggests CS-CuNPs as the most effective fungicide against rice sheath blight, one of the major threats to crop production in Vietnam. These findings are in line with several scientific studies and reports that support the application of nanotechnology as a new fungicide in sustainable agriculture and food security.

Conclusion

In the present study, CS-CuNPs were successfully produced using chitosan as a protector, ascorbic acid, and $NaBH_4$ as an ion copper de-salter. The product has a spherical or elliptical shape with a nanoscopic size (70 – 74 nm). Moreover, the fungicidal effect of CS-CuNPS against three plant pathogenic fungi, including *M. oryzae*, *P. capsici*, and *R. solani*, has been proven. The results indicate CS-CuNPs as a new and effective fungicide with high potential for applications in crop protection and agriculture.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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