Synthesis, characterization and evaluation of copper nanoparticles as agrochemicals against *Phytophthora* spp.

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***Abstract*—By using water as a solvent, copper nanoparticles (CuNPs) have been synthesized from copper sulfate via chemical reduction method in the presence of trisodium citrate dispersant and polyvinylpyrrolidone (PVP) as capping agent. The effects of the experimental parameters such as the concentration of reducing agent ($NaBH_4$), reaction temperature, molar ratio of citrate/Cu^{2+} and weight percentage ratios of Cu^{2+}/PVP on the CuNP sizes were studied. The size of CuNPs in a range of 3±1 nm was obtained at $NaBH_4$ concentration of 0.2 M, 50°C, citrate/Cu^{2+} molar ratio of 1.0 and Cu^{2+}/PVP weight percentage of 5%. The colloidal CuNPs were characterized by using UV–Visible spectroscopy, transmission electron microscopy (TEM), and X-ray diffraction (XRD) techniques. The colloidal solution of CuNPs (3±1 nm) was investigated the potential against *Phytophthora* spp. which cause economically crop diseases. Under *in vitro* test conditions, the inhibition of *Phytophthora* spp. mycelia growth at three concentrations of CuNPs (10, 20, 30 ppm) after 48 hours are 90.18%, 91.87% and 100%, respectively. These results provided a simple and economical method to develop the CuNPs-based-fungicide.**

***Index Terms*—antifungal activity, citrate dispersant, copper nanoparticles, *Phytophthora* spp., PVP**

Received: 12-11-2017; Accepted: 22-01-2018; Published: 31-12-2018

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1 INTRODUCTION

In recent years, nanoparticles have been extensively studied due to their unusual chemical and physical properties [1, 2]. The effective applications of the nanoparticles generally depend on their size, shape and protecting agents which could be controlled by the preparation conditions [3]. A number of different approaches to prepare metal nanoparticles such as Cu, Ag, Pt, Au have been reported. Some of these methods include photoreduction, chemical reduction using reducing agents in association with protecting agents [4-6].

Interestingly, the nanoparticles strongly exhibited the antifungal and antimicrobial activities [5, 6]. Among them, copper nanoparticles (CuNPs) have much attention. CuNPs showed a significant antifungal activity against various plant pathogenic fungi such as *Phytophthora*, *Corticium salmonicolor* [7]. *Phytophthora* is a genus of plant-damaging Oomycete whose member species are capable of causing enormous economic losses on crops. The genus *Phytophthora* approximately includes one hundred species [8]. *Phytophthora* spp. cause diseases such as blight, stem rots, fruit rots. Worldwide crop losses due to *Phytophthora* diseases are estimated to be multibillion dollars [9]. Synthetic chemicals are currently used for inhibiting this fungal growth. However, *Phytophthora* spp. are known to be able to develop the resistance to chemicals rapidly [10]. Thus, the discovery of new alternatives with lower risk of resistance plays a major role for controlling the pathogens as *Phytophthora* spp.

As mentioned, CuNPs showed a significant antifungal activity against *Phytophthora*. In addition, the cost to produce CuNPs is much cheaper than the others such as silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs). However, the studies on antifungal activity of CuNPs have not yet received much attention in Vietnam. The low cost to prepare the CuNPs is an advantage to use them in agriculture as agrochemicals. In this study, CuNPs were prepared in water by chemical reduction method in the presence of the sodium citrate dispersant and polyvinylpyrrolidone (PVP) as protecting agents. The effects of the experimental parameters such as the concentration of reducing agent ($NaBH_4$), reaction temperature, molar ratio of citrate/Cu^{2+} and the weight percentage ratios of Cu^{2+}/PVP on the size of the CuNPs were investigated. UV–Visible spectroscopy, transmission electron microscopy (TEM) and X-ray diffraction (XRD) techniques were used to characterize CuNPs. The antifungal activity against the growth of *Phytophthora* spp. mycelia was estimated under *in vitro* conditions on Potato Dextrose Agar (PDA) medium.

2 MATERIALS AND METHODS

Materials

Copper (II) sulfate ($CuSO_4$, 99.0%), polyvinylpyrrolidone (Mw 58,000 g/mol), trisodium citrate dihydrate ($HOC(COONa)(CH_2COONa)_2.2H_2O$, 99.0%), sodium borohydride ($NaBH_4$, 98%) were purchased from Acros Organics. All reagents were used without further purification. Distilled water was used as a solvent. *Phytophthora* spp. were supplied by Laboratory Applications in Microbiology, Institute of Tropical Biology, Vietnam Academy of Science and Technology, Linh Trung, Thu Duc, Ho Chi Minh City.

Synthesis of CuNPs

The mixture including PVP (0.2 g), $CuSO_4$ and $HOC(COONa)(CH_2COONa)_2.2H_2O$ was dissolved in 30 mL water. The mixture was heated and stirred for 5 minutes. The Cu^{2+} ions in the reaction mixture were then reduced to copper metal by the introduction of $NaBH_4$. As thermal reduction proceeded, the blue solution turned to red, indicating the formation of the CuNPs for 10 minutes.

Product characterization

UV–Vis absorption spectrum of the CuNPs solution was measured by Jasco V670 (Jasco Analytical Instrument). The colloidal CuNP solutions were diluted in water with the same concentrations prior to measuring UV-Vis spectra. The UV-Vis spectra were scanned in a wavelength range from 500 to 800 nm. TEM images were measured by JEM-1400 version (JEM-1400, JEOL). The samples for TEM measurement were prepared by dropping CuNPs solution onto a carbon-coated copper grid. The histogram of the particle-size distribution and the average diameter were obtained by measuring particles. The XRD result was characterized using D8 advanced Bragg X-ray (D8 Advance, Brucker) with Cu Kα radiation. For sample handling, glass slide was used as a substrate for measurement. Leaned substrate was covered with the colloidal CuNPs solution and dried in air.

Determination of the antifungal activity

The antifungal activity against *Phytophthora* spp. was estimated by using the *in vitro* plate dilution method. The colloidal CuNP solutions with various concentrations (10, 20, 30 ppm) were mixed with melting PDA medium to obtain a 15 mL total volume in Petri dishes. The control dishes contained 50 ppm of PVP, or 50 ppm of copper sulfate without colloidal CuNPs. The fungus *Phytophthora* spp. strain was activated by inoculation the mycelia on PDA dish at 37^{o}C, 72 hours. Then, the activated fungus was split into small pieces (5 mm x 5 mm). The treatments were performed by putting the small piece of active fungus in central of petri plates, wrapped with parafilm and incubated at 37^{o}C. The diameters of the colony growth of the control and CuNP samples were observed after 24 and 48 hours. Each treatment for each concentration of CuNPs was replicated three times. The inhibition of the growth of the mycelia was estimated by measuring the colony diameter and calculated by formula: growth inhibition (%) = (d1−d2/d1) × 100, where d1 and d2 are colony diameters of the control and CuNPs contained samples, respectively

3 RESULTS AND DISCUSSION

Characterization of CuNPs

The formation of CuNPs is confirmed by the powder X-ray diffraction (XRD). Figure 1 showing the peak positions with high crystallinity at 43.2^{o}, 50.4^{o}, and 74.0^{o} in XRD pattern are consistent with metallic copper. These peaks correspond to the typical face-centered cubic of copper with miller indices at (111), (200), and (220) which are in good agreement with the literature values [5, 11-14]. This result also indicated that copper oxides (Cu_2O and CuO) were not formed in the synthetic process. Furthermore, the color of solution had changed from blue to reddish. This observation revealed that the efficiency of reduction of copper salt (Cu^{2+}, blue) into copper metal (Cu^{0}, reddish) was significant.

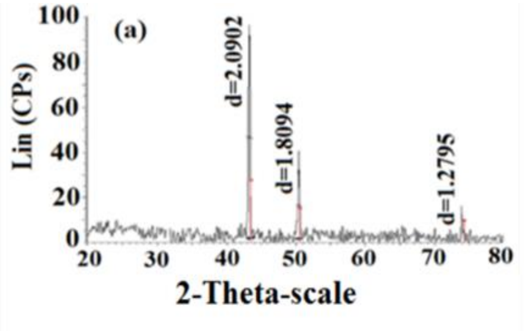

Fig 1. X-Ray diffractogram of CuNPs

Effect of reducing agent concentration on the size of copper nanoparticles: Sodium borohydride ($NaBH_4$) was used as a reducing agent to prepare CuNPs. Reaction temperature ($50^{o}C$), and the amount of PVP (0.2 g) were kept constant. The amounts of copper salt and trisodium citrate were used to ensure that the weight percentage ratio of Cu^{2+}/PVP and the molar ratio of citrate/Cu^{2+} were always 3% and 0.5, respectively. The reaction time was 15 minutes. The reducing agent of $NaBH_4$ allows a variation of concentration within a range of 0.1 – 0.5 M. Figure 2 illustrated the UV-Vis spectra of colloidal CuNP solutions with various concentrations of $NaBH_4$. The results showed that the surface plasmon resomance of CuNPs shifted to shorter wavelengths with increasing the $NaBH_4$ concentration (from 583 nm at 0.1 M to 570 nm at 0.2 M). However, the maximum absorption peaks shifted to longer wavelengths (574, 576 and 582 nm) at higher concentrations (0.3, 0.4 and 0.5 M) of reducing agent.

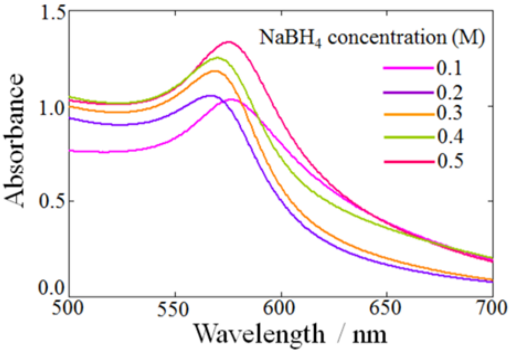

Fig. 2. UV-Vis spectra of colloidal CuNP solutions at various concentrations of $NaBH_4$

This observation could be attributed to an increase the CuNP sizes at higher concentrations (> 0.2 M) [5, 6, 18]. The CuNPs were generated in soltution through two stages. The copper nuclei is firstly generated and then was the growth of CuNPs [18]. It is thus important to control the preparation process that copper nuclei must generate faster and grow up slower. With increasing the concentration of $NaBH_4$, the reaction conversion rate of copper sulfate increased, the amount of copper nuclei rose, and small particle size powders were obtained. However, an excess number of copper nuclei would be generated when the reducing agent concentration was high. This resulted in an agglomeration of nuclei and a growth of the particle size. Thus, the optimal concentration of $NaBH_4$ was 0.2 M.

Effect of reaction temperature on the size of copper nanoparticles

Concentration of $NaBH_4$ (0.2 M), the weight percentage ratios of Cu^{2+}/PVP (3%) and the molar ratio of citrate/Cu^{2+} (0.5) were fixed in the experiments. The reaction temperature was varied in a range from 40 to 80°C. The UV-Vis spectra of colloidal CuNP solutions were given in Figure 3. The positions of maximum peaks had a decrease in wavelength with increasing the reaction temperature from 40 to 60°C (586 nm at 40°C, 575 nm at 50°C and 570 nm at 60°C). However, an increase in temperature from 60 to 80°C, the opposite shifts was obtained (575 nm at 70°C and 580 nm at 80°C). The results could be attributed to the change of CuNP sizes with varying the temperature. The CuNP size decreased when the temperature grew up to a certain range. The nucleation rate was greater than the growth rate when the temperature increased within a range from 40 to 60°C. At higher temperatures (> 60°C), the viscosity of the solution decreased and the growth rate enhanced due to CuNP collisions. As a result, the size of CuNPs increased with the increasing the reaction temperature [6, 18, 19]. At lower temperatures, the formation of CuNPs was not favorable. Therefore, the optimal reaction temperature was selected at 50°C.

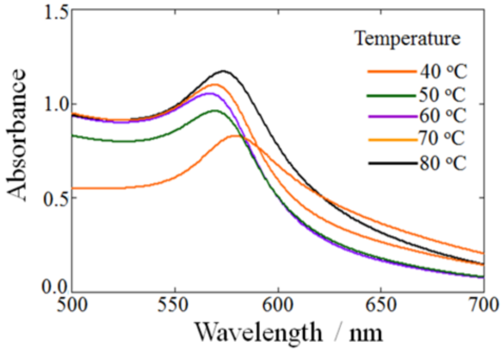

Fig. 3. UV-Vis spectra of colloidal CuNP solutions at different temperatures

Effect of citrate/copper salt ratio on the size of copper nanoparticles

In order to investigate the effect of citrate/Cu^{2+} ratios on the size of CuNPs, the reaction mixture was conducted at 50 °C, the concentration of reducing agent ($NaBH_4$) was 0.2 M, and the amount of PVP was 0.2 g while the molar ratio of citrate/Cu^{2+} varied in a range from 0.0 to 2.0. Figure 4 depicted UV–Vis absorption spectra of colloidal CuNP solutions in a range of molar ratio of citrate/Cu^{2+} from 0.0 to 2.0. In the presence of citrate dispersant, the maximum peaks of CuNPs shifted to shorter wavelength with increasing molar ratio of citrate/Cu^{2+}. This observation could result from a change in nanoparticle size. However, absorbed wavelengths (≈ 567 nm) were insignificantly different when using molar ratios ranging from 1.0 to 2.0.

Effect of the weight percentage ratio of copper salt to PVP on the size of copper nanoparticles

During the synthetic process, the reaction temperature (50°C) and the reducing agent concentration (0.2 M), and the amount of PVP (0.2 g) were kept constant. The weight percentage ratios of Cu^{2+} to PVP were varied in a range from 1 to 13%. The amount (mole) of citrate was also varied according to the variation of the copper salt amount in solutions so that the molar ratio of citrate/Cu^{2+} (1.0) was always constant. The UV-Vis spectra of the CuNP solutions were given in Figure 5. The results showed that the absorbance at maximum peaks increased with increasing the weight percentage ratio of Cu^{2+} to PVP from 1 to 11%. The position of the maximum peaks in a region of 567–570 nm. When the Cu^{2+}/PVP ratio reached 13%, the peak shifted to longer wavelength (573 nm). This result showed that the size of CuNPs increased at the Cu^{2+}/PVP ratio of 13%.

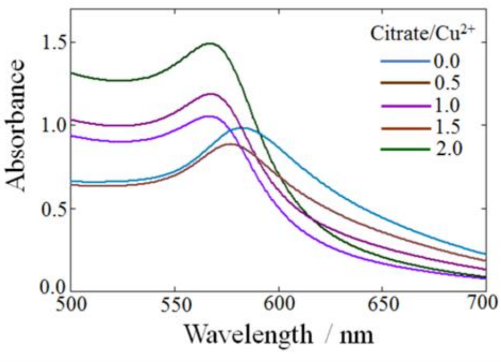

Fig. 4. UV-Vis spectra of the colloidal CuNP solutions with different molar ratios of citrate/copper salt.

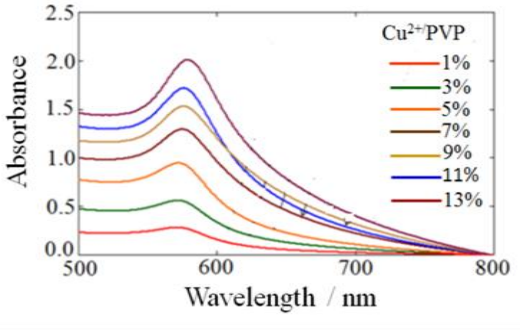

Fig. 5. UV–Vis spectra of colloidal CuNP solutions with various weight percentage ratios of Cu^{2+} to PVP.

TEM images of samples

The TEM images of CuNPs shown in Figure 6 confirmed the correlation between the citrate concentration and the size of the produced CuNPs. The changes of size caused the UV-Vis spectra to shift to shorter wavelengths as mention above. In the absence of citrate, average diameter of CuNPs was in a range of 20±7 nm, whereas its diameter appeared in a range of 3±1 nm at molar ratio of citrate/Cu^{2+} of 1.0. Thus, this ratio of citrate to copper salt was optimized at 1.0 to prepare CuNPs for biological tests. Moreover, these results confirmed that CuNPs with smaller sizes absorb at shorter wavelengths in UV–Vis spectra.

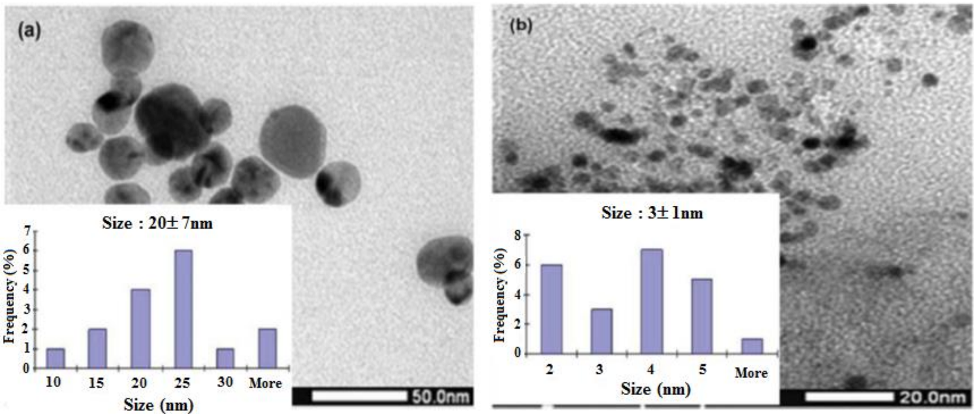

Fig. 6. TEM images of CuNPs prepared in the absence (a) and the presence (b) of citrate dispersant. The molar ratio of citrate/Cu^{2+} is 1.0

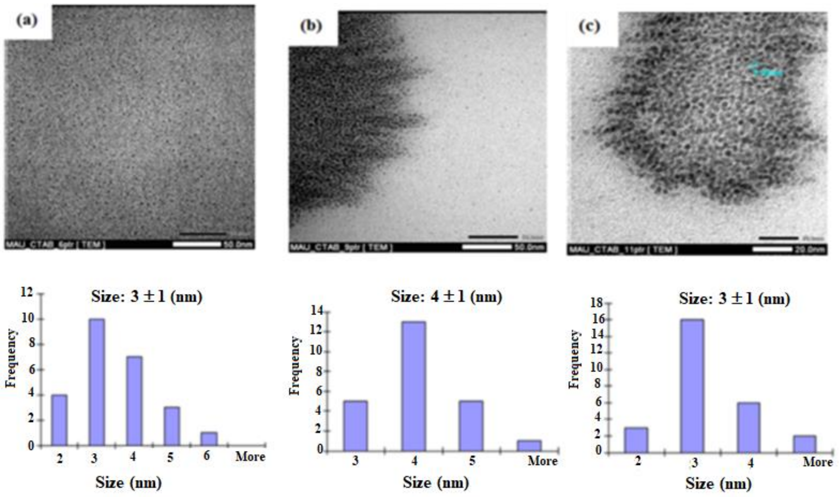

Fig. 7. TEM images of CuNPs prepared in various ratios of Cu^{2+}/PVP: (a) 5%, (b) 9% and (c) 11%

Figure 7 showed the TEM images and size distribution of the copper nanoparticles which were synthesized with different of ratio of Cu^{2+}/PVP. At ratio of Cu^{2+}/PVP=5% (Fig. 7a), the copper nanoparticles were synthesized mainly in spherical, uniform distribution with the size 3 ± 1 nm. When the ratio of Cu^{2+}/ PVP increased to 9 % and 11% (Fig. 7b, 7c), the copper nanoparticles were prepared in an approximate spherical shape, higher concentration and cluster formation because of high concentration of nanoparticles formed. However, due to the synergistic of PVP and citrate, the nanoparticles were prepared with small size, the size in range of 4±1 nm and 3±1 nm, respectively.

Synergistic effect of citrate dispersant and PVP capping agent: Polyvinylpyrrolidone (PVP) has been extensively used as a capping polymer to protect colloidal solution containing metallic nanoparticles [5, 6]. However, the bulky polymer is ineffective to coat all surfaces of the metallic nanoparticles. These results in an outgrowth in size of particles were due to their collision. To prevent this disadvantage, a molecular protecting agent like trisodium citrate could be used. A certain amount of trisodium citrate molecules is adsorbed on the surface of metallic nanoparticles. As a consequence, the aggregation of nanoparticles due to their collision was significantly reduced. Furthermore, it has been hard to prepare these small and uniform-sized metallic nanoparticles in the sole presence of capping polymers or citrate dispersant [6]. The synergistic effect of citrate dispersant and capping polymer has been expected to control size growth of CuNPs as demonstrated in Figure 8. Citrate and PVP work as size controller and polymeric capping agents, because they hinder the nuclei from aggregation through negative charge and polar groups, which strongly absorb the CuNPs on the surface via electrostatic interactions coordination bonds [15, 16].

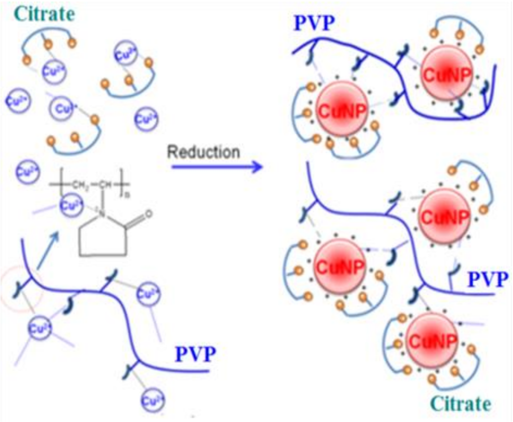

Fig. 8. A demonstration of the synergistic effect of citrate dispersant and PVP capping polymer on controlling size growth of CuNPs. Left figure depicts the formation of complexes of copper ions and citrate or PVP. The synergistic effect of citrate and PVP is given in the right figure.

Stability of colloidal CuNP solutions: The colloidal CuNP solutions were synthesized by using optimized conditions described above. Figure 9 showed that the positions of peaks at maximum absorption wavelengths (569 nm) were not changed after 1 and 3 months of storage. This observation confirmed that the CuNPs were stable during storage time, i.e., the size of CuNPs was not changed. However, the maximum peaks shifted to 579 nm after 5 and 6 months. This result revealed that the size of CuNPs increased with increasing the storage time. Furthermore, there was no peak of Cu_2O at 450 nm, i.e., the CuNPs were not oxidized during the storage period.

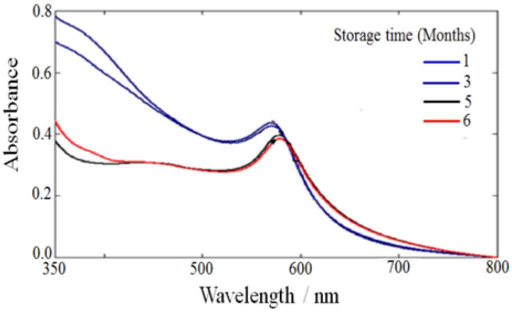

Fig. 9. UV-Vis spectra of the colloidal CuNP solutions at different storage times

CuNPs inhibit *Phytopthora* spp. *in vitro*

Basing on optimized experiments above, the CuNPs having an average diameter in range of 3±1 nm were prepared. The potential of the colloidal CuNP solutions at various concentrations (10, 20, 30 ppm) were estimated against *Phytophthora* spp. Figure 10 showed the antifungal ability against *Phytophthora* spp. After 48 hours of the incubation, the highest antifungal activity was observed at the CuNP concentration of 30 ppm (100%). At lower concentrations of 10 ppm and 20 ppm, CuNPs were less effective with 90.18% and 91.87% of fungal growth inhibition, respectively.

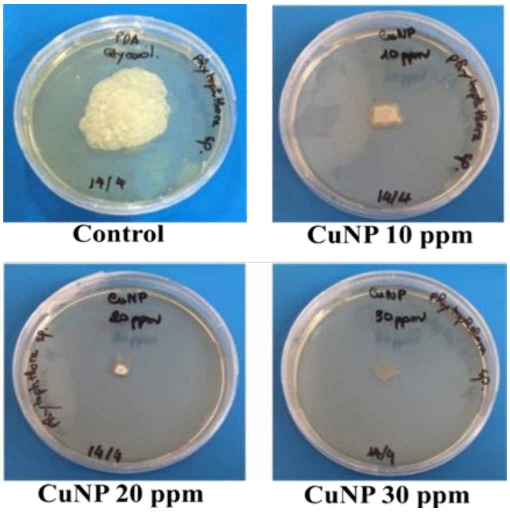
Control
CuNP 10 ppm
CuNP 20 ppm
CuNP 30 ppm

Fig. 10. The fungal growth inhibition of CuNPs at various concentrations against *Phytophthora* spp. after 48 hours of incubation. CuNPs were not added to control

Nanoparticles can be currently used as alternatives to chemical pesticides. Most of CuNP studies have focused on antibacterial activities and to a lesser extent on antifungal activities. Under *in vivo* condition, the chromosomal DNA degradation in *E. coli* started within 30 minutes of treatment with CuNPs, and more degradation occurred with the increasing of the nanoparticle exposure time. The mechanism of antibacterial activity of CuNPs in *E. coli* cells has been proposed. The copper ions (Cu^{2+}) attributed to be the main effector for DNA degradation, the nascent ions were generated from the oxidation of metallic CuNPs when they were in the vicinity of agents, namely cells, biomolecules or medium components [17]. To the best of our knowledge no study has been reported to explore the mechanism of the growth inhibition of CuNPs on *Phytophthora* spp.

4 CONCLUSION

CuNPs were prepared via chemical reduction method under the presence and the absence of citrate dispersant and PVP capping polymer. The purity and stability of the CuNPs were revealed by X-ray diffraction (XRD), UV–Vis spectroscopy and TEM techniques. The effects of the concentration of reducing agent, the reaction temperature and the ratios of copper salt to protecting agents on the CuNP sizes were investigated. In order to obtain a small size distribution (3±1 nm), the experimental conditions were optimized. The optimal concentration of $NaBH_4$, and reaction temperature were 0.2 M and 50°C, respectively. The ratios of Cu^{2+} to citrate (citrate/Cu^{2+}-molar ratio) and PVP (Cu^{2+}/PVP-weight percentage) were 1.0 and 5%, respectively. In solution, citrate and PVP played as size controller and capping agents, they impeded the aggregation of CuNPs by forming coordination bonds via negative charge and polar groups. The CuNPs having the size of 3±1 nm were estimated the inhibition of the fungal growth and exhibited a high potency of the antifungal against *Phytophthora* spp. under *in vitro* treatments. The result showed a complete inhibition of the *Phytophthora* spp. mycelia growth at 30 ppm. This result demonstrated that CuNPs not only were used as alternatives to chemical pesticides against *Phytophthora* spp. without any phytotoxicity but also can be applied as a novel antifungal agent in agriculture to control the plant pathogenic fungi.

Acknowledgment: *The authors are thankful to Lac Hong University and Ho Chi Minh City University of Technology and Education for support. Laboratory Applications in Microbiology, Institute of Tropical Biology, Vietnam Academy of Science and Technology, Linh Trung, Thu Duc, Ho Chi Minh City is gratefully acknowledged.*

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Tổng hợp, xác định cấu trúc hóa học và khảo sát hoạt tính kháng nấm *Phytophthora* spp. của hạt đồng nano

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Ngày nhận bản thảo: 12-11-2017; Ngày chấp nhận đăng: 22-01-2018; Ngày đăng: 31-12-2018

***Tóm tắt*—Các hạt đồng nano (CuNPs) đã được tổng hợp trong dung môi nước bằng phương pháp khử hóa học trong sự hiện diện của các tác chất phân tán trisodium citrate và chất bảo vệ polyvinylpyrrolidone (PVP). Ảnh hưởng của nồng độ chất khử ($NaBH_4$), nhiệt độ phản ứng, tỷ lệ mol citrate/Cu^{2+} và tỷ lệ khối lượng Cu^{2+}/PVP lên kích thước các hạt đồng nano đã được khảo sát. Kích thước 3±1 nm của các hạt đồng nano đạt được tại nồng độ chất khử là 0,2 M, nhiệt độ phản ứng là 50°C, tỷ lệ mol citrate/Cu^{2+} là 1,0 và tỷ lệ khối lượng Cu^{2+}/PVP là 5%. Đặc điểm hạt đồng nano được xác định bằng phổ tử ngoại-khả kiến (UV–Vis), chụp ảnh dưới kính hiển vi điện tử truyền qua (TEM) và nhiễu xạ tia X (XRD). Hoạt tính kháng nấm của các hạt đồng nano (kích thước 3±1 nm) được thử nghiệm đối với nấm *Phytophthora* spp. Thử nghiệm *in vitro* cho thấy, chế phẩm đồng nano tại các nồng độ 10, 20 và 30 ppm đã ức chế 90,18%, 91,87% và 100% sự phát triển của tơ nấm *Phytophthora* spp. sau 48 giờ. Kết quả này là cơ sở để phát triển chế phẩm diệt nấm đơn giản, kinh tế dựa trên các hạt đồng nano.**

***Từ khóa*—hoạt tính kháng nấm, chất phân tán citrate, hạt đồng nano, *Phytophthora* spp., PVP**