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Effect of oligochitosan-coated silver nanoparticles (OCAgNPs) on the growth and reproduction of three species *Phytophthora in vitro*

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ABSTRACT

Phytophthora diseases cause billions of dollars annually in damage to crops. Nanotechnology provides various metallic nanoparticles exhibiting a strong activity against microbial pathogens. Silver nanoparticles-based products are outstanding samples, which has been produced in large scale and performed well with a high activity against several fungal pathogens. Our previous study indicated oligochitosan-coated silver nanoparticles (OCAgNPs) which were prepared from 3,4 dihydroxyphenyl acetic acid – conjugated oligochitosan and silver nitrate salt performed an enhancement in antibacterial ability at a very low concentration. Objective of this study was to determine the effect of the OCAgNPs on growth and reproduction of *Phytophthora capsici*, *P. nicotianae* and *P. colocasiae in vitro*. The study shown that strong inhibition of mycelial growth, sporangium production, zoospore release and zoospore germination of *P. capsici*, *P. nicotianae* and *P. colocasiae* occurred when exposed to at 9 ppm of OCAgNP. The results demonstrated a great potential of OCAgNPs for controlling growth of *Phytophthora*.

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Antifungal activity; silver nanoparticles; *P. capsici*; *P. nicotianae*; *P. colocasiae*

1. Introduction

The genus *Phytophthora*, "plant destroyer", is one of the most destructive genera of plant pathogens causing many diseases on economically important plant species in tropical countries. It causes billions of dollars in damage annually as reported by Ribeiro (2013). For example, Granke et al. (2013) and Sanogo and Ji (2012) reported that *P. capsici* causes root, stem and fruit rot, leaf and stem blight and wilt and black pod on onion, sweet pepper, watermelon, citrus, cucumber, squash, pumpkins, tomato, eggplant, macadamia, black pepper and cacao. In addition,

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Phytophthora nicotianae attacks many crops such as pineapple, custard apple, jack fruit, areca palm, pawpaw, banksia, pea, orchid, lemon, grapefruit, orange, cotton, lettuce, tomato and tobacco and *Phytophthora colocasiae* is the causal agent of leaf spot and blight of taro (Ludowici et al. 2013; Miyasaka et al. 2013).

Metallic nanoparticles such as silver, copper and gold were reported to exhibit a strong activity against some microbes, pathogenic fungi and microorganisms (Kim et al. 2012; Zhou et al. 2012; Cao et al. 2014, 2015; Cu et al. 2014). Metallic silver, in the form of silver nanoparticles, has been reported as a potential antimicrobial agent (Yin et al. 2010; Zhou et al. 2012). As several pathogens have developed resistance against various antibiotics, this problem may be overcome by the use of silver nanoparticles according to Rai et al. (2009). For examples, silver and copper nanoparticles were used as antimicrobial activity against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* (Ruparelia et al. 2008). Silver nanoparticles (AgNPs) prepared by green synthesis approaches, showed high antimicrobial activity against Gram-positive and Gram-negative bacteria (Sharma et al. 2009). Silver-containing filters have been shown to have antibacterial properties in water and air purification. The use of AgNPs also exhibited antifungal agents against several plant pathogens. For example, Kim et al. (2012) found that silver nanoparticles inhibited 18 fungal pathogens that cause various diseases on vegetable, fruits and other crops.

Oligochitosan, obtained by hydrolysis or degradation of chitosan, is biodegradable, non-toxic and biocompatible. It possesses versatile functional properties as well as a potential plant immunity regulator. Plant diseases could be managed by using oligochitosan, which activates plant immunity that includes signal perception and transduction and accumulation of induced defence-related secondary metabolites (Yin et al. 2010) (Kim and Rajapakse 2005).

To date, there have not been many studies on the effects of silver and oligochitosan on *Phytophthora* spp. in Vietnam. Our previous study indicated that OCAgNPs significantly inhibited growth of *E. coli* and *S. aureus* at a very low concentration of AgNPs (2.5 ppm) (Cu et al. 2014). The high antibacterial activity of the NPs was explained that an increment of electrostatic affinity between the positive-charged NPs and negative-charged bacterial membrane resulted in enhancing penetration of the NPs into the bacteria. The objective of this study was to determine the *in vitro* effects of the Oligochitosan-coated silver nanoparticles on growth and reproduction of three *Phytophthora* spp.

2. Materials and methods

2.1. Culture

Phytophthora capsici, *P. nicotianae* and *P. colocasiae* were isolated from black pepper, pomelo (Thanh Tra crop) and taro, respectively. The morphology of three species was showed in Figure 1. Culture isolates were stored in sterile water at

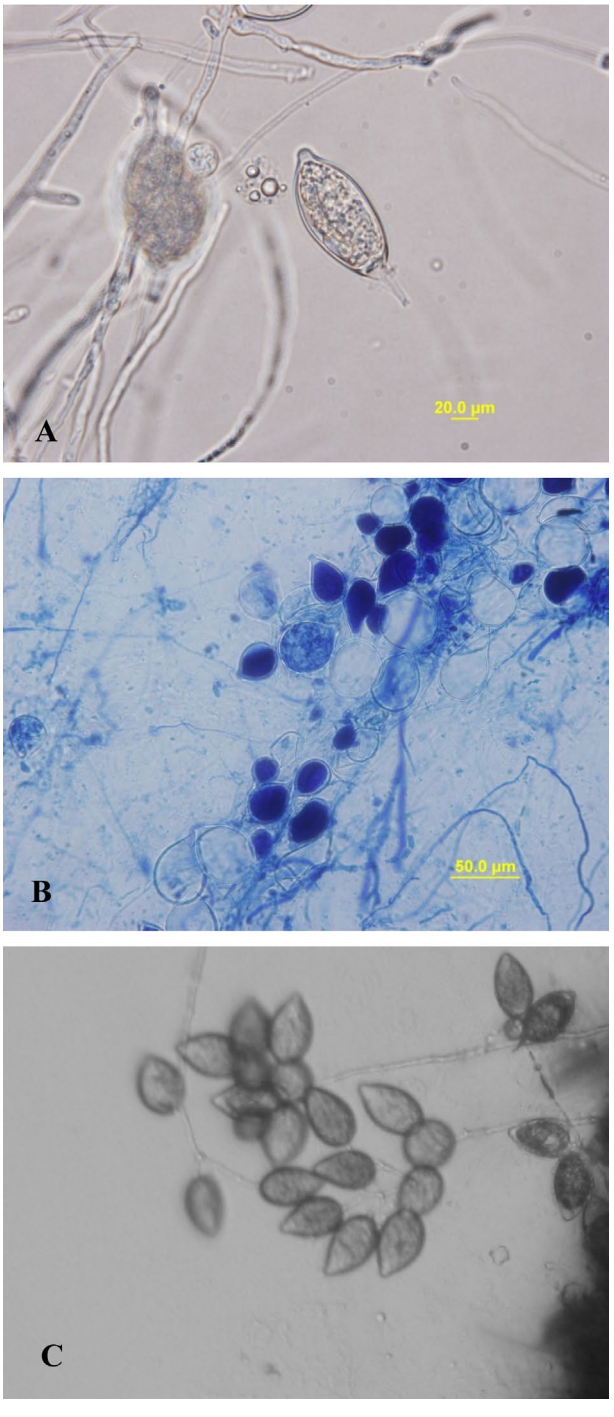

Figure 1. Morphology of three Phytophthora species: A. *P. capsici*, B. *P. nicotianae*, C. *P. colocasiae*.

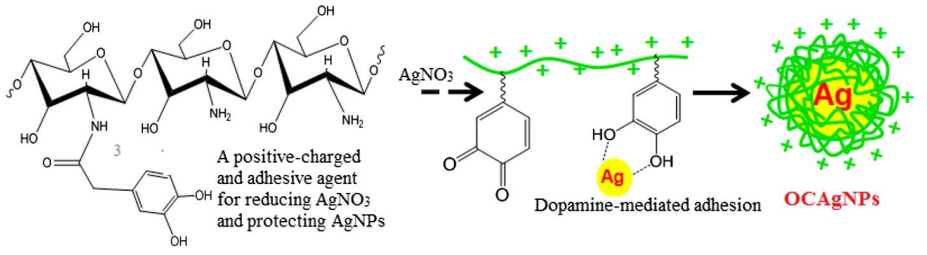

Figure 2. Demonstration of OCAgNPs synthetic process.

the Department of Plant Protection, School of Agriculture and Forestry, Hue University. This study was carried out at the School of Agriculture and Forestry, Hue University from 2014 to 2016.

2.2. Preparation of OCAgNPs

In the study, 3,4-dihydroxyphenyl acetic-conjugated oligochitosan, a reductant agent was prepared from oligochitosan and 3,4-dihydroxyphenyl acetic acid in the presence of carbodiimide coupling reagent (Cu et al. 2014). Silver core-DHPAC shell NCs were prepared by mixing 50 ml DHPAC solution (1%) the containing around 1600 ppm of DHPA and 50 ml $AgNO_3$ solution containing 800 ppm of silver ion and then stirred for 30 min at room temperature (Cu et al. 2014). The Ag core-DHPAC shell NCs could be formed (as demonstrated in Figure 2) and characterised using TEM and XRD.

2.3. Effect of OCAgNPs on mycelial radical growth, sporangium production, on zoospore release and cystospore germination of Phytophthora species

Potato dextroseagar (PDA) was prepared in Schott bottle and autoclaved at 121 °C for 30 min. After the medium cooled to 55 °C, OCAgNPs prepared as described above, was amended to the medium for final concentrations of 0, 3, 6 and 9 ppm and then was poured into Petri dishes (90 mm diameter). Mycelial discs (5 mm diameter) of the individual *Phytophthora* sp. grown on PDA medium were cut from the margins of the colony and placed on the OCAgNPs-amended PDA plates. After 2 days incubation in the dark at 25 °C, the diameter of each colony was measured daily. Growth inhibition was calculated by the Abbott formula (Chen et al. 2015) (%) = [(C− T)/C] × 100, where C and T are the diameter of the colony on the non-amended control and OCAgNPs-amended medium, respectively. All experiments were performed in three replicates. One plate was used for each concentration.

Carrot agar (CA) was prepared in Schott bottle and autoclaved. After the medium cooled to 55 °C, silver oligochitosan was amended to the medium for final concentrations of 0, 3, 6 and 9 ppm and then was poured into Petri dishes

(90 mm diameter). The mycelium of the various *Phytophthora* spp. were grown on CA in the dark condition for 5 days and then transferred under continuous light for another 5 days. To collect sporangia, sterile distilled water was added to each dish, and a glass spreader was used to rub off the surface of the media. One plate was used for each concentration. The resulting suspension containing sporangia was decanted into a 50 ml tube. The concentration of sporangia in the suspension was estimated using a hemacytometer. The counting was repeated fifteen times for each concentration.

Carrot agar (CA) amended with OCAgNPs was prepared as described above. Cultures of the *Phytophthora* spp. were grown in Petri dishes containing the non-amended and amended CA in the dark for 5 days at the room temperature and then transferred under continuous light for another 5 days. The plates were first flooded, then the sporangia were detached by using a glass rod and the suspension decanted into a 50 ml tube. One plate was used for each concentration. The tube were exposed to 4 °C for 60 min and then returned to room temperature under continuous light to stimulate the release of zoospores. The concentration of zoospores released was estimated with a hemacytometer. The counting was repeated fifteen times for each concentration.

Water agar (2%; WA) amended with OCAgNPs was prepared as described above. Sporangia and zoospores were produced on carrot agar (CA) medium as described above. One plate was used for each concentration. An aliquot (5 µl) of zoospore suspension was placed on WA media not amended (0 ppm) or amended with OCAgNP. Germination of cystospores was examined microscopically and the length of the germ tube was measured after 1 h at room temperature. The measure was repeated thirty times for each concentration.

2.4. Data analysis

The data collected on the diameter of colony growth, number of sporangia and zoospores and the length of the germ tube was analysed through one-way ANOVA using SPSS software v.16 (IBM Inc.). The numbers of sporangia and zoospores were transformed into square root before analysis. Treatment means were computed using Tukey's Multiple Comparison Test at $P \leq 0.05$.

3. Results

3.1. Characterisation of OCAgNPs

XRD diffractogram of the OCAgNPs performed crystalline phase of metallic silver at 38.0, 44.2, 64.4, 77.6 and 81.6º that corresponded to the typical face-centred cubic structure of Ag with miller indices at (111), (200), (220), (311) and (222), respectively, as seen in Figure 3(A).

OCAgNPs were produced in which size distribution of Ag cores ranging from 10 to 30 nm that was covered with polymer shell layer as seen in Figures 3(B)

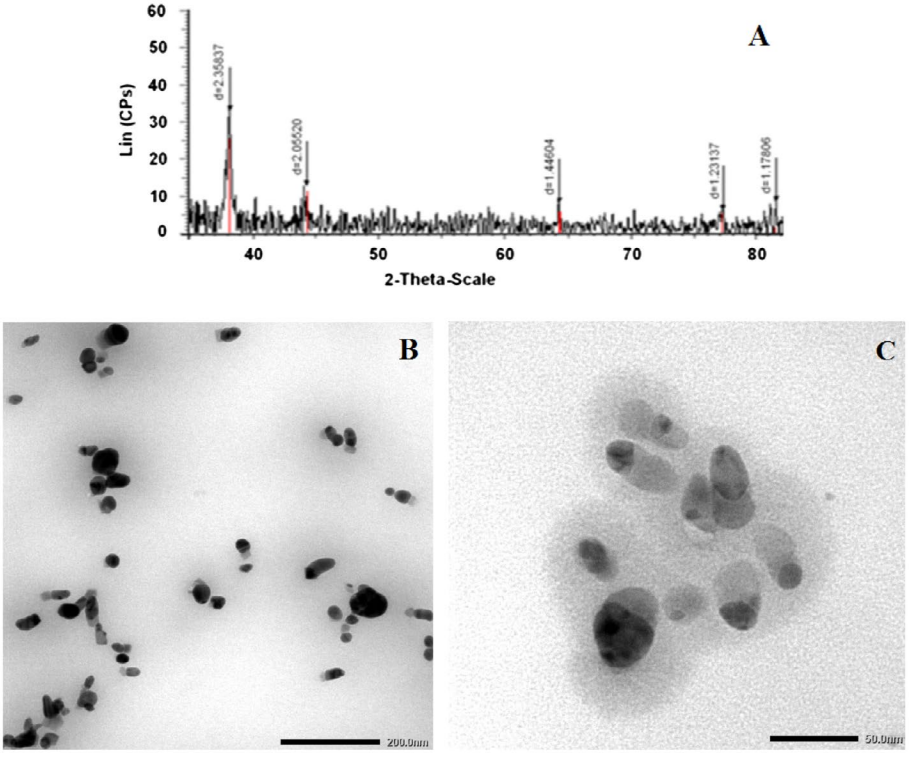

Figure 3. XRD partern (A) and TEM images of the OCAgNPs (B and C).

and 2(C). These obtained results confirmed that the OCAgNPs were synthesised at high uniformity and purity.

3.2. Effect of OCAgNPs on mycelial growth of Phytophthora *species*

The effect of the OCAgNPs on mycelial growth of the three *Phytophthora* spp. is shown in Table 1. The colony diameter decreased within four days 4 as the OCAgNPs concentration increased from 0 to 9 ppm. Tukey test analysis indicated that there was a significant difference in colony diameter regardless of the species tested on PDA amended with OCAgNPs with different concentration of 0, 3, 6 and 9 ppm ($P \leq 0.05$). Mycelial growth inhibition was from 33.1 to 79.4%, 31.2 to 77.2% and 49.4 to 62.0% for *P. capsici*, *P. nicotianae* and *P. colocasiae*, respectively (Figure 4 and 5).

3.3. Effect of OCAgNPs on sporangium production of Phytophthora *species*

The effect of OCAgNPs on sporangia production is shown in Table 2 and Figure 6. The number of sporangia decreased as the OCAgNPs concentration increased from 0 to 9 ppm. Tukey test analysis indicated that there was a significant difference in the number of sporangia on CA amended with OCAgNPsconcentration

Table 1. Mycelial growth of three species of *Phytophthora* on potato dextrose agar medium amended with various concentrations of OCAgNPs.

OCAgNPs concentration (ppm)	Diameter of colony(mm)[1]											
	After day 3			After day 4			After day 5			After day 6		
	P. cap[2]	*P.nic*	*P. col*	*P.cap*	*P.nic*	*P. col*	*P.cap*	*P.nic*	*P. col*	*P.cap*	*P.nic*	*P. col*
0	41.6[a]	41.3[a]	28.3[a]	56.0[a]	53.0[a]	34.3[a]	67.3[a]	71.6[a]	43.0[a]	79.3[a]	77.0[a]	52.6[a]
3	28.3[b]	22.0[b]	13.0[b]	36.0[b]	29.3[b]	16.3[b]	47.0[b]	44.6[b]	23.6[b]	53.0[b]	53.0[b]	26.6[b]
6	23.6[c]	17.6[b]	13.3[b]	28.6[c]	24.0[c]	16.0[b]	34.6[c]	29.0[c]	22.3[b]	44.0[c]	38.6[c]	24.3[b]
9	10.6[d]	11.6[c]	8.6[b]	12.0[d]	14.0[d]	11.0[b]	14.6[d]	15.3[d]	16.6[c]	16.3[d]	21.0[d]	20.0[c]

Notes: [1]Mean diameter of colony was measured at 3, 4, 5 and 6 days after incubation in the dark at 25 °C. Treatment means were compared using Tukey's Multiple Comparison Test. Means followed by different letters in the same column differ significantly ($P \leq 0.05$).
[2]*P. cap* = *Phytophthora capsici*, *P. nic* = *P. nicotianae*, *P. col* = *P. colocasiae*.

of 0, 3, 6 and 9 ppm ($P \leq 0.05$). Inhibition of sporangia production was from 54.0 to 90.9%, 66.3 to 91.4% and 33.3 to 82.1% for *P. capsici*, *P. nicotianae* and *P. colocasiae*, respectively.

3.4. *Effect of OCAgNPson zoospore release of* Phytophthora *species*

The effect of the OCAgNPs on zoospore release is shown in Table 3. The number of zoospore decreased as the OCAgNPs concentration increased from 0 to 9 ppm. Tukey test analysis indicated that there was a significant difference in the number of zoospore on CA amended with OCAgNPs of 0, 3, 6 and 9 ppm ($P \leq 0.05$). Inhibition of zoospore release was from 38.5 to 85.0%, 44.9 to 90.6% and 31.1 to 79.7% for *P. capsici*, *P. nicotianae* and *P. colocasiae*, respectively.

3.5. *Effect of OCAgNPs on cystospore germination of* Phytophthora *species*

The effect of the OCAgNPs on germ tube length is shown in Table 4. The length of the germtube decreased as the OCAgNPs concentration increased from 0 to 9 ppm. Tukey test analysis indicated that there was a significant difference in the length of the germtube on WA amended with OCAgNPs of 0, 3, 6 and 9 ppm ($P \leq 0.05$). Inhibition of zoospore germination was from 25.9 to 100.0%, 10.8 to 100.0% and 68.1 to 100.0% for *P. capsici*, *P. nicotianae* and *P. colocasiae*, respectively.

4. Discussion

This *in vitro* study showed that OCAgNPs inhibit mycelial growth, sporangia formation, zoospore release and zoospore germination in three selected species of *Phytophthora*. Vegetative and reproductive structures produced by species of *Phytophthora* are important in the epidemiology of diseases caused by these pathogens (Sanogo and Ji 2012, 2013). Management tools that reduce the production of these structures should reduce the impact of diseases incited by species of

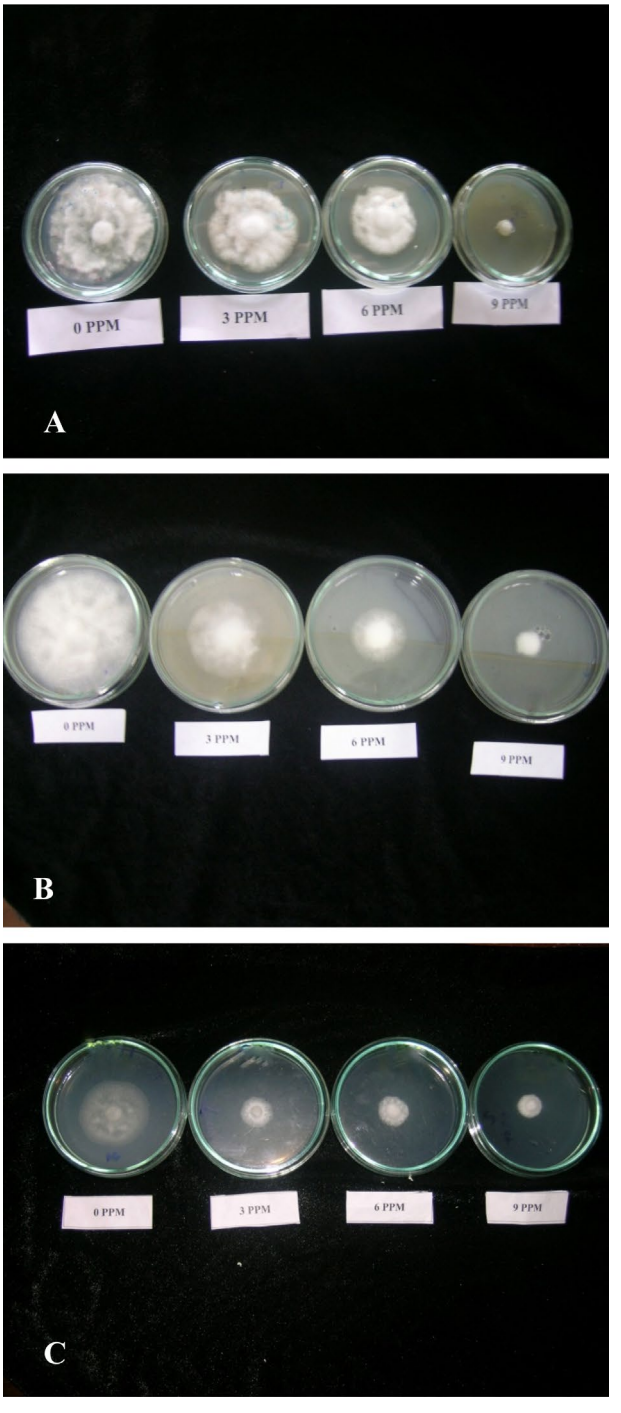

Figure 4. Mycelial growth of three Phytophthora species after six days of incubation on PDA amended with different concentration OCAgNPs (0, 3, 6, 9 ppm): A. *Phytophthora capsici*, B. *P. nicotianae*, C. *P. colocasiae*.

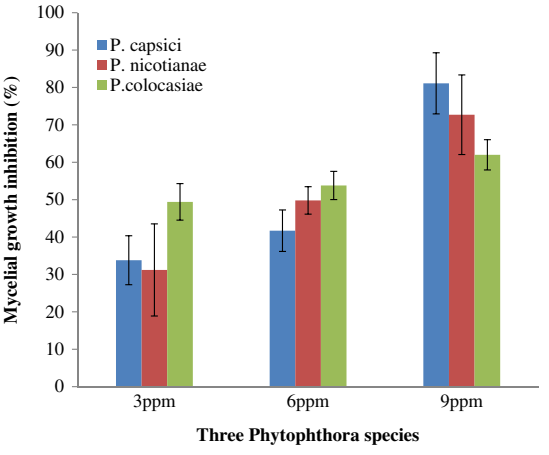

Figure 5. Mycelial growth inhibition in three species of *Phytophthora* on potato dextrose agar medium with various concentrations of OCAgNPs after incubation in dark at 25 °C for 6 days.

Table 2. Production of sporangia by three species of *Phytophthora* on carrot agar medium amended with various concentrations of OCAgNPs.

OCAgNPs concentration (ppm)	*P. capsici*		*P. nicotianae*		*P. colocasiae*	
	NS[1] (sporangium/mL)	EH[2] (%)	NS (sporangium/mL)	EH (%)	NS (sporangium/mL)	EH (%)
0	$18.5\times10^{4a}\pm273.0$	–	$13.0\times10^{4a}\pm356.1$	–	$16.0\times10^{4a}\pm88.4$	0
3	$8.7\times10^{4b}\pm107.9$	52.8	$4.4\times10^{4b}\pm112.3$	66.3	$10.6\times10^{4b}\pm76.4$	33.3
6	$3.1\times10^{4c}\pm22.8$	82.9	$2.0\times10^{4c}\pm75.4$	84.3	$5.5\times10^{4c}\pm90.6$	65.6
9	$1.7\times10^{4d}\pm115.1$	90.6	$1.1\times10^{4c}\pm54.9$	91.4	$2.8\times10^{4d}\pm101.6$	82.1

Notes: [1]Mean number of sporangia (NS) five days after incubation in the dark at 25 °C. Numbers following " ± " are the standard error of the mean. Treatment means were compared using Tukey's Multiple Comparison Test. Means followed by different letters in the same column differ significantly ($P \leq 0.05$).
[2]EH = Effect of inhibition.

Phytophthora. This study indicates that OCAgNPs has the potential to minimise plant infection by *Phytophthora* species. Silver nanoparticles (AgNPs) have been reported as antimicrobial agents against various pathogens including bacteria, fungi and virus (Jose Ruben et al. 2005; Quang Huy et al. 2013; Rajeshkumar and Bharath 2017; Khan et al. 2018). AgNPs was reported that it could inhibit the pathogens through multifaceted mechanisms, lysis of bacterial cell wall and antioxidant, inhibition of transcription and translation process and antibiotics agents (Dakal et al. 2016; (Patra and Baek 2017; Rajeshkumar and Bharath 2017). In addition, AgNPs have low phytotoxicity and a broad spectrum of antimicrobial activity against various plant fungal pathogens (Kim et al. 2012). Mycelial growth was inhibited in 18 different plant pathogenic fungi grown on potato dextrose agar, malt extract agar and corn meal agar amended with AgNPs.

Oligochitosan have been reported as an antifungal agent to *Phytophthora* species (Vasyukova et al. 2001; Falcon et al. 2007). The mechanism of action of oligochitosanwas studied by Xu et al. (2007a) showed high antifungal activity

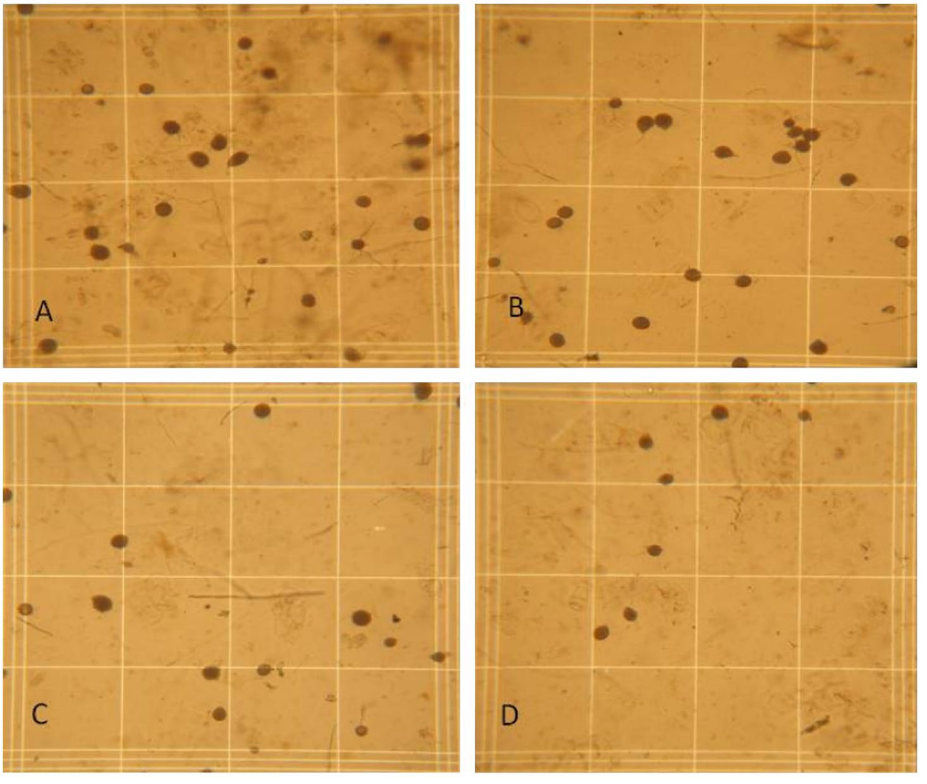

Figure 6. Effect of OCAgNPs at four concentrations on sporangium production by *Phytophthora capsici* A: 0 ppm; B: 3 ppm; C: 6 ppm ; D: 9 ppm OCAgNPs.

Table 3. Production of zoospores by three species of *Phytophthora* on carrot agar medium amended with various concentrations of OCAgNPs.

OCAgNPs concentration (ppm)	*P. capsici*		*P. nicotianae*		*P. colocasiae*	
	NZ[1] (spore/mL)	EH[2] (%)	NZ (spore/mL)	EH (%)	NZ (spore/mL)	EH (%)
0	$12.0\times10^{4a}\pm74.4$	–	$25.1\times10^{4a}\pm288.1$	–	$11.8\times10^{4a}\pm81.8$	–
3	$7.3\times10^{4b}\pm93.3$	39.1	$13.8\times10^{4b}\pm389.1$	44.9	$8.1\times10^{4b}\pm60.5$	31.1
6	$2.7\times10^{4c}\pm104.6$	77.0	$10.6\times10^{4b}\pm226.4$	57.5	$5.0\times10^{4c}\pm53.7$	57.6
9	$1.8\times10^{4c}\pm204.6$	85.0	$2.3\times10^{4c}\pm123.7$	90.6	$2.4\times10^{4d}\pm123.7$	79.7

Notes: [1]Mean number of zoospores (NZ) at five days after incubation in the dark at 25 °C. Numbers following " ± " are the standard error of the mean. Treatment means were compared using Tukey's Multiple Comparison Test. Means followed by different letters in the same column differ significantly ($P \leq 0.05$).
[2]EH = Effect of inhibition.

against *P. capsici* by disruption of cell endomembrane system. Oligochitosan was also found to be more effective than chitosan in inhibiting mycelial growth and different stages in the life cycle of *P. capsici* as observed by Xu et al. (2007b). Oligochitosan caused the rupture of released zoospores induced. An ultrastructural study indicated that oligochitosan caused distortion and disruption of most vacuoles, thickening of the plasmalemma and appearance of unique tubular materials. Plasmalemmasomes in the hyphal tip cells were not found in the presence of

Table 4. Germination of zoospores in three species of *Phytophthora* on water agar medium amended with various concentrations of OCAgNPs.

OCAgNPs concentration (ppm)	*P. capsici*		*P. nicotianae*		*P. colocasiae*	
	GL[1] (µm)	EH[2] (%)	GL (µm)	EH (%)	GL (µm)	EH (%)
0	$11.2^{a} \pm 1.0$	–	$18.73^{a} \pm 0.8$	–	$16.6^{a} \pm 1.1$	–
3	$8.3^{b} \pm 0.6$	25.9	$5.97^{b} \pm 0.4$	10.8	$14.8^{a} \pm 0.6$	68.1
6	$6.9^{b} \pm 0.6$	38.4	$3.19^{c} \pm 0.2$	43.4	$9.4^{b} \pm 0.3$	83.0
9	$0.0^{c} \pm 0.0$	100.0	$0.0^{d} \pm 0.0$	100.0	$0.0^{c} \pm 0.0$	100.0

Notes: [1]Mean Germ tube length (GL) was measured at 1 h after incubation of cystospores in the dark at 25 °C. Numbers following " ± " are the standard error of the mean. Treatment means were compared using Tukey's Multiple Comparison Test. Means followed by different letters in the same column differ significantly ($P \leq 0.05$).
[2]EH = Effect of inhibition.

oligochitosan. The polycationic nature of oligochitosan contributes only partly to its antifungal activity and multiple modes of action of oligochitosan exist including the disruption of the endomembrane system.

Our previous study indicated that oligochitosan are cationic polysaccharide played a role as an active targeting molecule as coated on the AgNPs. The nanosystem could induce electrostatic interaction with phospholipid layer on microbial membrane that contributes to enhance its antimicrobial property (Cu et al. 2014).

In this study, it was found that OCAgNPs was capable of inhibiting three *Phytophthora* spp. that are commonly found in Vietnam at concentrations varying between 3 and 9 ppm. However, in most of fungi inhibition was found at high concentration of 100 ppm (Kim et al. 2012). The increase in inhibition with an increase in the OCAgNPs concentration is in agreement with the finding reported by Kim et al. (2012).

Management of Phytophthora diseases of horticulture crops and fruits is a challenge to farmers. This study shows the potential for an economically important and environmentally friendly alternative. In the past few years, there has been an increased great effort to develop safe management methods that pose less harm to human health and the environment, with a focus on overcoming dependencies on chemical fungicides. The effect of the OCAgNPs on the growth and reproduction of the tested *Phytophthora* spp. indicate that OCAgNPs has a potential as an antifungal nanomaterial. This product inhibited different stages in the life cycle of *Phytophthora* species. This work opens the future prospects for use of the OCAgNPs in the management of Oomycete pathogens. However, the current study is based on invitro petri dish evaluation; therefore, the signification of these finding to more general cases is limited. To validate the *in vitro* finging, further investigation with OCAgNPs should be conducted *in vivo* with plant treatment with the nanoparticles and plant inoculation with species of *Phytophthora.* Among other issues, the *in vivo* study should address the method, number and timing of applications of nanoparticles. Results from the *in vivo* study could be used to guide the development of nanoparticles for commercial use.

5. Conclusion

AgNPs are known as one of the most attractive nanomaterials that could be commercialised as an environmentally friendly fungicide or an ecofungicide. This *in vitro* study indicates that a low concentration of the oligochitosan-coated siver nanoparticles produced by VAST can provide protection against plant pathogens such as species of *Phytophthora* species. This study provides an increase in the knowledge of the antifungal effects of AgNPs in the management of plant diseases. Additionally, this study will help researchers to uncover the mechanisms of the antifungal activity of AgNPs against Oomycetes pathogens. Thus, a new theory on the fungicidal properties of nano-size silver colloidal solution for controlling various Oomycete pathogens has been developed in this study. However, field investigations are needed to demonstrate that AgNPs that can be applied in planta to manage plant diseases.

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Disclosure statement

Finances to conduct these studies were contributed mainly from the authors. The authors declare that there is no conflict of interests regarding the publication of this paper.

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