Inhibition of *Phytophthora parasitica* and *P. capsici* by Silver Nanoparticles Synthesized Using Aqueous Extract of *Artemisia absinthium*

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ABSTRACT

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Application of nanoparticles for controlling plant pathogens is a rapidly emerging area in plant disease management, and nanoparticles synthesis methods that are economical and ecofriendly are extensively investigated. In this project, we investigated the potential of silver nanoparticles (AgNPs) synthesized with aqueous extract of *Artemisia absinthium* against several *Phytophthora* spp., which cause many economically important crop diseases. In in vitro dose-response tests conducted in microtiter plates, 10 $\mu\text{g ml}^{-1}$ of AgNPs inhibited mycelial growth of

P. parasitica, *P. infestans*, *P. palmivora*, *P. cinnamomi*, *P. tropicalis*, *P. capsici*, and *P. katusae*. Detailed in vitro dose-response analyses conducted with *P. parasitica* and *P. capsici* revealed that AgNPs synthesized with *A. absinthium* extract were highly potent (IC₅₀: 2.1 to 8.3 $\mu\text{g ml}^{-1}$) and efficacious (100%) in inhibiting mycelial growth, zoospore germination, germ tube elongation, and zoospore production. Interestingly, AgNP treatment accelerated encystment of zoospores. Consistent with in vitro results, in planta experiments conducted in a greenhouse revealed that AgNP treatments prevented *Phytophthora* infection and improved plant survival. Moreover, AgNP in in planta experiments did not produce any adverse effects on plant growth. These investigations provide a simple and economical method for controlling *Phytophthora* with AgNP without affecting normal plant physiology.

Silver nanoparticles (AgNPs) display strong antibacterial, antifungal, and antitumor activities (Kharissova et al. 2013). Due to their excellent antimicrobial activities and desirable physico-chemical properties, AgNPs are currently extensively investigated for application in various industries including medicine, diagnostics, cosmetics and food processing (Thorley and Tetley 2013, Project on Emerging Nanotechnologies 2013). In fact, they are already used in wound dressings, food packaging and in consumer products such as textiles and footwears for fighting odor-causing microorganisms (Schluesener and Schluesener 2013; Velmurugan et al. 2014; Project on Emerging Nanotechnologies 2013). AgNPs are primarily composed of zerovalent silver (Ag⁰) clusters, which typically range in size from 5 to 100 nm in diameter. Depending on their synthesis chemistry, AgNP preparations may consist of nanospheres, nanotubes, triangular crystals, or a combination of these shapes. The three-dimensional nano structures of AgNPs are stabilized by various capping agents primarily biopolymers such as cellulose, pectin, guar gum and polyethylene glycol (George et al. 2014; Lavorgna et al. 2014; Mandal et al. 2012; Raghavendra et al. 2013). Multiple mechanisms have been proposed for the antimicrobial properties of AgNPs. They display high affinity for sulfur and phosphorus. Their interaction with sulfur containing amino acids inside or outside the cells affects cell viability (Prathna et al. 2011). Another possible mechanism includes the release of silver ions from AgNPs and their subsequent interaction with phosphorus in DNA, thereby inactivating DNA replication. The released silver ions can also react with sulfur-containing proteins, leading to inhibition of enzyme and protein functions (Gupta 1998). Additionally,

silver ions have been reported to inhibit respiratory chain proteins and interfere with membrane permeability (Holt and Bard 2005; Shrivastava et al. 2007). Since AgNPs displays multiple modes of inhibitory action against microorganisms (Clement and Jarrett 1994), the chances of pathogens developing resistance to AgNPs are minimized. Due to their diminished resistance development, AgNPs may be used for controlling fungicide resistant plant pathogens more effectively.

Silver nanoparticles synthesized using plant extracts have been shown to inhibit plant pathogenic bacteria and fungi (Kaur et al. 2012; Kim et al. 2012; Panacek et al. 2009; Pimprikar et al. 2009). To our knowledge, however, no study has reported the potential of AgNP against oomycetes, which are biologically different from true fungi. The oomycete genus *Phytophthora* includes more than one hundred species, which infect a wide range of food, feed and ornamental crops in managed agriculture and forest trees in natural ecosystems (Kroon et al. 2012). Worldwide crop losses due to *Phytophthora* diseases are estimated to be multibillion dollars (Wawra et al. 2012). Prominent examples include \$6.7 billion losses in potato due to late blight (Haverkort et al. 2008) and \$1 to 2 billion in soybean due to *Phytophthora* root rot (Tyler 2007). Some of the most destructive and well-known *Phytophthora* species are *P. infestans* (late blight of potato and tomato), *P. parasitica* (blight, and root and stem rots in annual ornamentals and trees including citrus), *P. capsici* (blights in numerous vegetables), *P. sojae* (soybean root and stem rot), and *P. ramorum* (sudden oak death) (Cacciola and Lio 2008; Cline et al. 2008; Erwin and Ribeiro 1996; Gevens et al. 2007; Grunwald et al. 2012; Kroon et al. 2012). Currently, various synthetic chemicals are used for controlling these pathogens. However, *Phytophthora* spp. are known to develop resistance to chemicals very rapidly (Childers et al. 2015; Dobrowolski et al. 2008; Gisi and Cohen 1996; Hu et al. 2012; Hu et al. 2005; Hu et al. 2008, 2010; Hwang and Benson 2005; Meng et al. 2011; Perez-Sierra et al.

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2011; Randall et al. 2014; Timmer et al. 1998). Fungicide resistance is one of the major problems in managing diseases caused by *Phytophthora* spp. Addressing fungicides resistance would require discovery of alternative products with new mode of actions. AgNPs, due to their potentially lower risk of resistance development, could play a major role in fungicide resistance management of *Phytophthora* spp.

AgNPs can be synthesized using various physical methods such as plasma catalysis and laser ablation (Amendola et al. 2007) and chemical methods which require a reducing agent such as sodium borohydrate (Borase et al. 2014; Zhu et al. 2000). Physical methods involve high temperature and lasers, which are expensive and require specialized equipment and skilled personnel. Chemical synthesis methods usually involve high temperatures and pressure, and potential production of hazardous by-products raise environmental concerns (Borase et al. 2014; Zhu et al. 2000). Green synthesis, using biological material as a source of reducing agent and capping agent, offers comparatively safer and eco-friendly approach for nanoparticles synthesis (Borase et al. 2014). Numerous biological sources including extracts from plant tissues and microbes have been employed for the synthesis of AgNPs (reviewed in Borase et al. 2014; Kharissova et al. 2013). Although the exact mechanism of how biological materials mediate AgNP synthesis is not well understood, various metabolites and enzymes are suggested to provide reducing potential for reducing Ag⁺ to Ag⁰. In addition to reducing agents, biological extracts are also suggested to provide capping agents, which stabilize AgNPs (reviewed in Borase et al. 2014; Kharissova et al. 2013). *Artemisia absinthium* L. is naturally found in the foothills of Himalayas in the Indian subcontinent. Previously, our group, as well as others, have shown that this plant species possesses strong antioxidant activity, potentially providing an excellent source for reducing Ag⁺ to AgNP (Ali and Abbasi 2014a, b; Ali et al. 2013; Lee et al. 2013; Singh et al. 2012). Using aqueous extract of *A. absinthium*, we have successfully synthesized and characterized AgNP (Ali et al., unpublished data). In this report, we investigated the potential of these AgNPs in inhibiting *Phytophthora* spp. To our knowledge, this is the first report showing effective control of *Phytophthora* spp. using silver nanoparticles. The major objectives of this study were (i) to evaluate efficacy and potency of *Artemisia*-mediated AgNP against different *Phytophthora* spp. in vitro and in planta, and (ii) to determine the effect of AgNP on various developmental and reproductive stages of *Phytophthora* spp. Findings of our investigations will expand the repertoire of products that can be used either alone or rotated with other chemicals for controlling *Phytophthora* diseases.

MATERIALS AND METHODS

Synthesis of silver nanoparticles. Greenhouse-raised *A. absinthium* plants were dried at room temperature and used for AgNPs synthesis. Plant extract was prepared by boiling 1 g of the dried powdered leaves of *A. absinthium* in 10 ml of deionized

water for 5 min. The aqueous extract was cooled down to room temperature (25°C), filtered through a 0.45 µm filter (Millex) and stored at 4°C until used. For the synthesis of silver nanoparticles, AgNO₃ (2 mM) and aqueous plant extracts prepared as described above were mixed in equal volumes, and reactions were allowed to progress at room temperature for 24 h. Unreacted AgNO₃ and plant extracts were removed by pelleting and washing AgNPs as follows. Reaction mixtures were centrifuged at 14,000 × g for 10 min at room temperature. Supernatants were discarded and the AgNP pellets were resuspended in deionized water followed by centrifugation at 14,000 × g for 10 min. This process was repeated five times. The resulting AgNP were resuspended in deionized water, and used in antimicrobial assays.

AgNP synthesis was monitored by recording UV-vis spectra (λ 250 to 700 nm) using either a NanoDrop 2000C Spectrophotometer (Thermo Fischer Scientific) or the Synergy H1 Hybrid multimode microplate reader (BioTek).

In vitro *Phytophthora* inhibition assays and data analyses. Antimicrobial potency and efficacy of AgNPs were assayed against various *Phytophthora* spp. in vitro. Sources of *Phytophthora* spp. used as targets in this report are provided in Table 1. However, most of the inhibition assays were focused on *P. parasitica* and *P. capsici*. Initially, effects of 10-fold dilutions of AgNPs (100, 10, and 1 µg ml⁻¹) were tested against different *Phytophthora* spp. In vitro tests were performed in a high throughput microtiter plate assay as described before (Ali and Reddy 2000). Briefly, assay mixtures were assembled in a 96-well flat-bottom microtiter plate with each well containing 10% V8 juice, 3,000 zoospores and a series of twofold AgNP dilutions (100 to 0.10 µg ml⁻¹, wt/vol) in a 200 µl total reaction volume. Controls were without AgNP. Each treatment was replicated four times, and experiments were repeated at least three times. Microtiter plates were wrapped with parafilm and incubated at 25°C in a humid chamber for maintaining high humidity. Optical density (OD₆₀₀ nm) of microtiter plates was read immediately and 24 h after the start of experiments with the Synergy H1 hybrid multimode microtiter plate reader (BioTek). Net growth was determined by subtracting OD₆₀₀ nm data at the start of experiment from the data at the 24-h interval. Net growth data were normalized to untreated control, and analyzed using the four-parameter sigmoidal logistic models using the Prism 6.0 software (GraphPad Software, Inc.). Statistical analyses of goodness-of-fit of curves were also performed using Prism 6.0. IC₅₀ values, AgNP concentration required for 50% growth inhibition, were calculated from the fitted logistic curves. Plates were examined under the microscope and pictures were recorded with a digital camera attached to an inverted microscope (IX8, Olympus).

To evaluate the effect of AgNP on zoospore germination, germ tube length, and zoospore encystment, zoospores were treated with twofold AgNP dilutions (100 to 0.10 µg ml⁻¹, wt/vol) in microtiter plates as described above. Additionally, sporangial production and the subsequent zoospore release were monitored regularly for several days. Each treatment was replicated three times. Fifteen minutes after AgNP treatments, swimming and encysted zoospores were counted in three fields of view under an inverted microscope (IX8, Olympus). Ten hours later, three random pictures were taken in each well of the microtiter plate, and germinated and ungerminated zoospores were counted. Germ tube lengths were measured using the NIH Image J software. Dose-response data on zoospore germination, germ tube length and zoospore encystment were fitted to a four-parameter sigmoidal curve using the Prism 6.0 software (GraphPad Software, Inc.). IC₅₀ values for the above parameters were calculated from the fitted sigmoidal curves.

In planta inhibition of *Phytophthora* by AgNP. For in planta *P. parasitica* inhibition assays, *Nicotiana benthamiana* (PI 555478) plants were grown from seeds in a greenhouse maintained at 25 ± 5°C with a16-h light/8-h dark photoperiod. After 15 to 21 days, *N. benthamiana* plants were transferred to a walking

TABLE 1. Growth inhibition of *Phytophthora* spp. by silver nanoparticles (AgNP)

<i>Phytophthora</i> sp.	Source	AgNP (µg ml ⁻¹) ^a		
		100	10	1
<i>P. parasitica</i>	Citrus	+	+	–
<i>P. capsici</i>	<i>Solanum lycopersicon</i>	+	+	–
<i>P. palmivora</i>	Spathiphyllum	+	+	–
<i>P. cinnamomi</i>	Azalea	+	+	–
<i>P. infestans</i>	<i>S. lycopersicon</i>	+	+	–
<i>P. tropicalis</i>	Ivy	+	+	–
<i>P. katsurae</i>	Palm	+	+	–

^a + indicates complete inhibition of mycelial growth compared with untreated control; – indicates no inhibition of mycelial growth.

growth chamber maintained at $25 \pm 5^\circ\text{C}$ with a 16-h light ($90 \mu\text{mol/s/m}^2$) and 8-h dark cycle. Plants were sprayed until runoff with the following treatments: AgNP at $100 \mu\text{g ml}^{-1}$, AgNP at $10 \mu\text{g ml}^{-1}$, mefenoxam (Subdue MAXX, $33.2 \mu\text{g}$ of active ingredient ml^{-1}), as a positive control and water as a negative control. Each treatment consisted of three replicates with each replication consisting of nine plants arranged in a 3×3 matrix in disposable plastic square pots (103 cm^2). One day later, treated plants were thoroughly sprayed with 25 ml of *P. parasitica* zoospore suspension (10^5 zoospores ml^{-1}). For zoospore suspension preparation, *P. parasitica* was grown for 2 weeks on solidified 20% V8 juice agar containing 0.2% CaCO_3 under dark at 25°C in a climate-controlled growth chamber. Sporangia were then scrapped off the plate in 5 ml of sterile water and incubated at room temperature (25°C) for 30 min to release zoospores. Concurrently, *P. parasitica* zoospores were treated with similar treatments in a microtiter plate for microscopic observations. Ten days postinoculation, number of healthy plants that survived and did not display any Phytophthora symptoms were counted. Data on percent healthy plants were calculated for each treatment, and statistically analyzed for significance of differences between treatments using Student's *t* test. Experiments were repeated two times.

RESULTS

Synthesis and characterization of AgNP by UV-vis spectroscopy. The change in the color of reaction mixture to yellowish brown or dark brown after mixing a plant extract and silver nitrate is a general characteristic of silver nanoparticle biosynthesis. Twenty-four hours after mixing *A. absinthium* aqueous extract with AgNO_3 , brown color colloidal solution of AgNP appeared (Fig. 1A). No color change was observed with the plant extract or AgNO_3 alone under the same conditions. UV-visible spectroscopy analyses also showed an increase in UV-vis spectrum above 350 nm with the most pronounced increase in the 400 to 500 nm range (Fig. 1B). AgNPs were physically characterized using transmission electron microscopy, energy dispersive X-ray, dynamic light scattering, and zeta potential (Ali et al., unpublished data).

AgNP inhibits *Phytophthora* spp. in vitro. Potential of AgNP to inhibit different *Phytophthora* spp. was evaluated in vitro at different vegetative and reproductive developmental stages. These included mycelial growth, zoospore germination and germ tube length (vegetative growth), and sporangial and zoospore release (reproduction), all of which determine pathogenicity and epidemic development. Effects of three AgNP concentrations (100 , 10 , and $1 \mu\text{g ml}^{-1}$) on mycelial growth of various economically important *Phytophthora* spp. were evaluated in vitro in microtiter plates. Microscopic examination of mycelial growth showed that AgNP applied at the 100 and $10 \mu\text{g ml}^{-1}$ rates strongly inhibited the growth of all *Phytophthora* spp. tested after 12 h of incubation (Table 1). Growth with the AgNP $1 \mu\text{g ml}^{-1}$ treatment was similar to control.

To determine potency and efficacy of AgNPs, twofold serial dilutions of AgNP ranging from 100 to $0.1 \mu\text{g ml}^{-1}$ were tested against *P. parasitica* and *P. capsici*. Dose-response data were fitted with a sigmoidal logistic curve, which indicated that AgNP inhibited *Phytophthora* mycelial growth in a dose-dependent manner (Fig. 2A and B). Goodness-of-fit analyses revealed very high R^2 and low sum of standard errors (Sy.x, indicated on Figure 2A and B), showing that parameter fits of sigmoid curves to the dose-response data of AgNP were significant. IC₅₀ and IC₉₀ values, which were calculated from the dose-response logistic curves, were very similar for *P. parasitica* and *P. capsici*, suggesting that AgNP inhibits both species equally well (Table 2). These observations were verified through microscopic examination of mycelial growths, which were consistent with quantitative data (Fig. 2C and D). Similarly, minimum inhibitory concentration

required for 100% inhibition for both *Phytophthora* species was $25 \mu\text{g ml}^{-1}$. Many antimicrobial compounds produce abnormal cell morphology such as irregular shaped hyphae, excessive branching, and hyphal blebbing. No such abnormal morphological characteristics were observed with any *Phytophthora* spp. treated with AgNPs.

AgNPs reduced zoospore germination, germ tube length, and sporangial production in *P. parasitica* and *P. capsici*. Successful infection of plants by *Phytophthora* is dependent on zoospore germination and germ tube elongation. Therefore, the effect of AgNP on these two pathogenicity parameters was investigated in vitro. Zoospore germination and germ tube length were both inhibited significantly by AgNP in a dose-dependent manner. Similar to mycelial growth, zoospore germination and germ tube length dose-response data also fitted typical sigmoidal curves with high goodness-of-fit values (Fig. 3A and B). IC₅₀ and IC₉₀ values for zoospore germination and germ tube lengths were comparable for both *Phytophthora* spp. (Table 2). Similarly *I_{max}* (maximum inhibition levels achieved) for mycelial growth, zoospore germination and germ tube lengths reached 100% with 10 to $25 \mu\text{g ml}^{-1}$ doses indicating that AgNP synthesized in the current study were highly efficacious against both *Phytophthora* spp. (Table 2).

Phytophthora epidemic developments are dependent on sporangial production and zoospore release. In in vitro tests, no sporangial production was observed for up to 15 days after AgNP treatments at the mycelial growth permissible rate ($\geq 6.25 \mu\text{g ml}^{-1}$). At lower AgNP concentrations ($\leq 3.12 \mu\text{g ml}^{-1}$), which did not inhibit

Fig. 1. Synthesis of silver nanoparticles using *Artemisia absinthium* extract. **A**, Glass vials showing silver nitrate (AgNO_3), *A. absinthium* aqueous extract and colloidal solution of silver nanoparticles (AgNPs) synthesized by mixing AgNO_3 and *A. absinthium* aqueous extract in a 1:1 ratio. **B**, UV-vis absorbance profiles (250 to 700 nm) of the above reaction mixtures after 24 h of incubation.

mycelial growth, sporangial production and zoospore production was not significantly different from the untreated controls (data not shown). Overall these results indicate that AgNP synthesized with *A. absinthium* extracts displayed strong activity against *P. parasitica* and *P. capsici*.

AgNP treatment enhances zoospore encystment in *P. capsici* and *P. parasitica*. After release from sporangia, *Phytophthora* zoospores swim around in the aqueous environment, often for several hours, to find appropriate infection sites. After doing so, zoospores encyst, germinate and penetrate host tissues. During routine microscopic examination of zoospores immediately after setting up AgNP treatment experiments, we noticed fewer swimming zoospores after treatment with higher concentrations of AgNP. This observation prompted us to hypothesize that AgNP might be accelerating zoospore encystment. To test this hypothesis, we analyzed zoospore encystment at several time points after treatment with different concentrations of AgNP. Compared with control, all zoospores ceased swimming within 10 min of treatment with AgNP at concentrations $\geq 10 \mu\text{g mL}^{-1}$. AgNP-treated zoospores assumed typical rounded shapes and sunk to the bottom of the microtiter plates rapidly. In contrast, swimming zoospores were observed in untreated controls for at least 4 h after the start of experiment. These results were further verified by statistically analyzing data on swimming and encysted zoospores after 15 min of

treatment with serial dilutions of AgNP (100 to $0.10 \mu\text{g mL}^{-1}$). Treatment with AgNP $\geq 1.56 \mu\text{g mL}^{-1}$ showed significantly higher zoospore encystment compared with control (Table 3). Significantly comparable results on zoospore encystment were recorded for *P. parasitica* and *P. capsici*. Dose-response data of encysted and swimming zoospores was fitted with sigmoidal curves (Fig. 3C).

TABLE 2. IC50 and IC90 values of silver nanoparticles (AgNPs) against *Phytophthora* spp.

<i>Phytophthora</i> sp.	IC50 ^a	IC90 ^b
<i>P. parasitica</i>		
Hyphal growth	8.2 (7.1–9.4)	15.9 (12.0–21.2)
Zoospore germination	2.1 (1.9–2.2)	4.1 (3.7–4.5)
Germ tube length	3.0 (2.4–3.8)	8.1 (4.5–14.8)
<i>P. capsici</i>		
Hyphal growth	8.3 (6.4–10.7)	19.6 (14.4–25.5)
Zoospore germination	2.5 (2.4–2.6)	3.7 (3.5–3.9)
Germ tube length	3.0 (2.7–3.4)	6.3 (4.7–8.3)

^a IC (inhibitory concentration) 50 and IC90 values ($\mu\text{g mL}^{-1}$), for 50 and 90% inhibition, were predicted from logistic curves fitted to the dose-response data.

^b IC90 values approximately corresponded to minimum inhibitory concentrations, which are minimum concentrations that completely inhibited growth.

Fig. 2. Mycelial growth inhibition of *Phytophthora parasitica* and *P. capsici* by silver nanoparticles (AgNPs). Dose-response curves displaying growth inhibition of **A**, *P. parasitica* and **B**, *P. capsici* in response to different concentrations of AgNP. Light micrographs showing mycelial growth of **C**, *P. parasitica* and **D**, *P. capsici* after 24 h of treatment with the indicated AgNP concentrations. Complete inhibition of both species was observed in response to $25 \mu\text{g mL}^{-1}$ of AgNP. Bar = 100 μm .

These analyses showed dose-dependent enhanced encystment of zoospores with IC50 of 1.22 (95% confidence intervals: 1.12 to 1.32) and IC90 of 2.79 (95% confidence intervals: 2.4 to 3.2). Expectedly, percent swimming zoospores reduced significantly with increasing concentrations of AgNP.

AgNPs inhibit *P. parasitica* in planta. To study the potential of AgNP to control disease caused by *P. parasitica* in planta, tobacco plants were sprayed with 100 or 10 µg ml⁻¹ of AgNPs, followed by inoculation with *P. parasitica*. Application with mefenoxam (*SubdueMaxx*, Syngenta), a commonly used fungicide against *Phytophthora*, and water were used as positive and negative control treatments, respectively. The same treatments were also performed in a microtiter plate to compare in planta disease control to in vitro growth inhibition. Microscopic data of the microtiter

plate and visual observations of the plants 5 days after *P. parasitica* infection are presented in Figure 4A. Percent surviving plants, which did not display any symptoms of *Phytophthora* infection, were statistically analyzed. These analyses showed that compared with negative control, which displayed 7.7% average plant survival, AgNP treatments applied at 100 and 10 µg ml⁻¹ displayed 96.3% and 77.8% survival of tobacco plants, respectively (Fig. 4B). In planta disease control with 100 µg ml⁻¹ of AgNP was comparable to *SubdueMaxx* (*P* = 0.42), whereas, AgNP at the 10 µg ml⁻¹ dose was about 23% less effective than *SubdueMaxx* (*P* = 0.008), but still substantially (70%) better than untreated control.

DISCUSSION

A. absinthium is an important medicinal plant that displays strong antioxidant activity (Ali and Abbasi 2014a, b; Ali et al. 2013). In this study, we showed that AgNPs synthesized with aqueous extract of this plant efficiently inhibit several agriculturally important *Phytophthora* spp. Many species in this genus cause destructive diseases in plants and they are notorious for developing resistance to fungicides and also for breaking down resistance genes by rapidly undergoing genetic mutations. Availability of AgNPs that may provide broad spectrum protection would provide alternative tools for controlling diseases caused by *Phytophthora* spp.

Use of chemical pesticides against plant microbial diseases poses various challenges such as environmental pollution and development of pesticide resistance in microbes. Therefore, investigation aimed at discovering alternatives to chemical pesticides against microbial diseases are highly desirable. Nanoparticles can be used as alternatives to chemical pesticides, and studies reporting the use of nanoparticles for controlling fungi under field conditions are appearing in the literature (Kaur et al. 2012; Panacek et al. 2009; Pimprikar et al. 2009). Most of these studies have focused on antibacterial and to a lesser extent on antifungal activities. To the best of our knowledge no study has been reported to explore the use of AgNP for controlling *Phytophthora* or any other oomycetes, which are phylogenetically very different from true fungi. In this study, data showed efficacy at much lower concentrations than 100 µg/ml against several *Phytophthora* spp. AgNPs were highly potent and efficacious at different life stages, including mycelial growth, sporangial production and zoospore germination making them an excellent choice for controlling *Phytophthora* diseases. In vitro MICs for AgNPs synthesized in this study were about 25 µg ml⁻¹. Using the same concentration, variable inhibitions (24.7 to 83.5%) were reported for synthetic AgNPs against several different fungi (Kim et al. 2012), suggesting that AgNP reported in our studies might be more effective. However, this difference could be attributed to the source of AgNP (biological versus chemical) or the difference in the target organisms (*Phytophthora* versus fungi).

Interestingly, treatment with AgNPs accelerated zoospore encystment in *P. parasitica* (Fig. 3C). However, in contrast to

Fig. 3. Effect of silver nanoparticles (AgNPs) on different developmental stages of *Phytophthora* spp. Dose-response curves showing inhibitory effect of different concentrations of AgNPs on zoospore germination and germ tube length of **A**, *P. parasitica* and **B**, *P. capsici*, and **C**, zoospore encystment of *P. parasitica*.

TABLE 3. Effect of silver nanoparticles (AgNP) on *Phytophthora parasitica* zoospores encystment

AgNP (µg ml ⁻¹)	% encysted zoospores	Significance (α = 0.05) ^a
100.00	100 ± 0	—
50.00	100 ± 0	—
25.00	100 ± 0	—
12.50	96.6 ± 1.2	***
6.25	94 ± 0.9	***
3.13	72.9 ± 2	***
1.56	31.6 ± 5.8	***
0.78	23 ± 3	n.s.
0.39	17.1 ± 3.3	n.s.
0.00	11.2 ± 2.3	—

^a *** indicates significantly different from the untreated control (*P* < 0.05); n.s. indicates means are not significantly different from control (*P* > 0.05).

normal zoospore development, which involves encystment, germination and germ tube elongation, growth and germination of AgNP-induced encysted zoospores was completely arrested, suggesting that AgNPs might be affecting normal developmental physiology of zoospores. Further investigations using genomic, molecular and ultrastructural studies are needed to gain insight into how AgNPs affect zoospore development.

A majority of antimicrobial studies employing nanoparticles have focused on in vitro studies in a highly isolated controlled environment, which does not represent the real world scenarios (Chernousova and Epple 2013; Prabhu and Poulouse 2012; Prasad et al. 2011; Rai et al. 2009; Rai et al. 2012). To be useful for practical application in the field, which is a highly complex system consisting of many physical factors such as temperature, humidity and light, and biological entities such as microbial communities associated with plants, it is imperative to evaluate bioactivity of AgNPs in planta. AgNP or any other nanoparticles operate at the nano scale, and their activity could be impacted by various reducing and oxidizing agents in the microenvironments at the plant-pathogen interface. In this study, AgNPs were very effective in controlling disease on tobacco plants inoculated with *P. parasitica* under greenhouse conditions. The in planta efficacy of AgNPs ($100 \mu\text{g mL}^{-1}$) against *Phytophthora* reported in this study was very similar to the efficacies of AgNP against diseases caused by true fungi such

as the anthracnose of pepper, and powdery mildew of cucumber and pumpkins in the field (Lamsal et al. 2011a, b). This concordance in efficacy against true fungi and *Phytophthora* spp., which are placed in separate kingdoms (Judelson 2007), suggest that AgNPs have broad spectrum antimicrobial activities and that they could simultaneously control multiple diseases.

We did not observe any adverse impact on the growth and anatomy of tobacco plants even at several fold higher concentrations than the minimum required for complete growth inhibition, indicating that AgNPs synthesized in this study are safe for plant application. These results are consistent with several studies that did not report any adverse impact of AgNPs when used at concentrations that controlled plant diseases (Lamsal et al. 2011a, b). However, our results are different from other reports, where phytotoxicity of chemically synthesized AgNPs have been reported (Gubbins et al. 2011; Kumari et al. 2009; Navarro et al. 2008; Stampoulis et al. 2009; Yin et al. 2012). Differences and similarities in results between our study and these reports could be due to different physical properties such as capping of AgNP formed using plant extracts or differences in the methods of assaying phytotoxicities (Stampoulis et al. 2009). Although, AgNP are used in many different consumer products, several reports indicate adverse impact of AgNP on mammalian cells (Ma et al. 2011), fish (Asharani et al. 2008), and crustaceans (Blinova et al. 2013). In light of the above inconclusive reports, future studies that systematically investigate ecotoxicity of different kinds of AgNPs should be conducted before they can be commercialized for plant protection against diseases.

Conclusions. The application of nanoparticles against plant pathogens is a newly developing area. In this study we showed that *A. absinthium* mediated AgNPs showed high potency against agriculturally important pathogens in the genus *Phytophthora*. In vitro treatments resulted in complete inhibition of *P. parasitica* and *P. capsici* at several developmental stages including spore germination, germ tube length elongation and spore encystment. More importantly, in planta application of AgNPs on tobacco plants prevented disease caused by *Phytophthora* without any phytotoxicity. Biosynthesis and utilization of AgNPs against *Phytophthora* may reduce use of expensive chemical pesticides.

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Fig. 4. In planta inhibition of *Phytophthora parasitica* on tobacco plants by silver nanoparticles (AgNPs). **A**, Tobacco plants treated with the indicated concentrations of AgNP and mefenoxam (SubdueMaxx) followed by inoculation with *P. parasitica*. The corresponding light micrographs of mycelia treated with AgNP in vitro are shown in upper row. Pictures were taken 5 days after inoculation. **B**, Bar graphs showing percent plant survival after *P. parasitica* inoculation in response to treatment with AgNP, mefenoxam (SubdueMaxx), and water (control). Compared with water control, plant survival was significantly increased by AgNP treatments (*, $P = 0.008$, $n = 27$; **, $P = 0.001$, $n = 27$).

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