

Effects of Colloidal Silver Nanoparticles on Sclerotium-Forming Phytopathogenic Fungi

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Effects of silver nanoparticles on the phytopathogenic fungal growth were investigated. Fungal phytopathogens, especially for sclerotium-forming species *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *S. minor*, were selected due to their important roles in survival and disease cycle. Tests for the fungal hyphal growth revealed that silver nanoparticles remarkably inhibit the hyphal growth in a dose-dependent manner. Different antimicrobial efficiency of the silver nanoparticle was observed among the fungi on their hyphal growth in the following order, *R. solani* > *S. sclerotiorum* > *S. minor*. Tests for the sclerotial germination growth revealed that the nanoparticles showed significant inhibition effectiveness. In particular, the sclerotial germination growth of *S. sclerotiorum* was most effectively inhibited at low concentrations of silver nanoparticles. A microscopic observation revealed that hyphae exposed to silver nanoparticles were severely damaged, resulting in the separation of layers of hyphal wall and collapse of hyphae. This study suggests the possibility to use silver nanoparticles as an alternative to pesticides for sclerotium-forming phytopathogenic fungal controls.

Keywords : sclerotia, silver nanoparticle, soil borne fungi

Several members of plant pathogenic fungi belonging to ascomycetes and basidiomycetes develop sclerotia, including *Sclerotinia sclerotiorum*, *S. minor*, and *Rhizoctonia solani*. Sclerotia are asexual, resting, and melanized structures in which vegetative hyphae become interwoven, aggregated, and dehydrated (Ayers and Adams, 1979). Sclerotium serves as a survival structure that can be observed on the infected plant tissues, soil and plant debris. Sclerotia also contribute to the increase of inoculum density of the fungal species. Sclerotium-forming fungal pathogens are wide-

spread in the world and cause many important diseases in a wide host range of plants. For example, sheath blight caused by *R. solani* is one of the destructive diseases of rice (*Oryza sativa* L.), causing significant yield losses in all rice-growing countries (Datta et al., 1999). Basal drop disease caused by either *S. sclerotiorum* or *S. minor* is also one of the most prevalent and serious diseases of lettuce (*Lactuca sativa* L.) worldwide (Sabbarao, 1998). A close correlation between disease incidence and sclerotia density was demonstrated (Dillard and Grogan, 1985; Imolehin and Grogan, 1980), indicating that sclerotia are major factors for disease dissemination and initiation. As primary survival structures of the pathogens, sclerotia exhibit longevity in soil and resistance to unfavorable abiotic factors such as heat, drought, and fungicide (Coley-Smith, 1979). Moreover, sclerotia play a key role in disease cycles, either infecting host plants through direct germination or producing new air-borne inoculi, ascospores through carpogenic development (Steadman, 1979; Tourneau, 1979).

Diverse disease management such as chemical methods and genetic controls has been used for the control of the diseases caused by sclerotium-forming fungi (Marcum et al., 1977). However, their broad host range and formation of sclerotia make it difficult to control diseases. Efforts to search for alternatives to pesticides have been persistently conducted because the overuse of pesticides causes ecological and environmental problems as well as harmful effects on human beings. Among many natural antibiotic compounds, silver or silver ions have long been used in many areas due to their strong antimicrobial activity against pathogenic microbes and non-toxicity to humans (Elchiguerra et al., 2005; Yeo et al., 2003). Silver ions are very reactive, which are known to cause the inhibition of microbial respiration and metabolism as well as physical damage (Bragg and Rannie, 1974; Thurman and Gerba, 1989). Also, it was suggested that silver ions intercalate bacterial DNA once entering the cell, resulting in no further proliferation (Feng et al., 2000). Recently, nanotechnology

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amplified the effectiveness of silver particles. A larger surface area of silver nanoparticles increases their contact with microbes or permeability into the cells. Development of silver nanoparticle has restored interests in their antimicrobial effects since the widespread use of modern synthetic antibiotics. Unfortunately, the antimicrobial activity of silver nanoparticles has been evaluated mostly against animal pathogens (Kawahara et al., 2000; Richards, 1981). The objectives of this study were to evaluate the antimicrobial effects of silver nanoparticles especially on sclerotium-forming phytopathogenic fungi.

Materials and Methods

Fungal species, culture conditions, and silver nanoparticles. Fungal species were obtained from Korean Agricultural Culture Collection (KACC). These included *Rhizoctonia solani* (AG-5, KACC number 40145), *Sclerotinia sclerotiorum* (KACC 41065), and *S. minor* (KACC 41066). The fungi were routinely grown on media including MEA (0.2% (w/v) malt extract, 1.5% (w/v) agar) medium at room temperature. For measurement of hyphal growth, agar plugs (4 mm in diameter) were obtained from the actively-growing edge of culture plates of fungi, inoculated in the center of MEA medium, and incubated for 7 days at 24°C. Silver nanoparticles (WA-CA-WA13B, Nanover™) were obtained from BioPlus Co., Ltd. (Korea). The silver nanoparticles dissolved in distilled water were utilized in this study. These nanoparticles have colloidal shapes with an average size of 4-8 nm.

Assay for sclerotial germination. Sclerotia were freshly obtained by incubating the fungal plates at room temperature without any treatment. Sclerotia (2-4 weeks old) were briefly washed with sterilized distilled water and rinsed with 70% alcohol. For testing the sclerotial germination growth, one sclerotium was placed in the center of MEA medium supplemented with either concentrations of the silver nanoparticles (Nanover™, BioPlus, Co., Korea) or an equal volume of water. The plates were incubated at 24°C, and used for the measurement of sclerotial germination growth. The sclerotial germination rate was determined by measuring the diameter of mycelial colonies.

Scanning electron microscopy (SEM). Fungal culture on MEA plates was sprayed with 5 ml of the silver nanoparticle solution (10 ppm). This specimen was fixed in 4% glutaraldehyde for 3 h and treated with 0.1 M cacodylate buffer for 1 h. After washing with distilled water, the specimen was dehydrated in a graded ethanol series up to 100%, critical-point dried, and gold-coated using an ion sputter-coater. The specimen was observed on a Hitachi S-

3500N scanning electron microscope at an accelerating voltage of 10 kV.

Results

Effect of silver nanoparticles on the fungal hyphal growth. First, we tested the hyphal growth of *R. solani*, *S. sclerotiorum*, and *S. minor* between several synthetic media including MEA, PDA (20 g potato dextrose, 1.5% agar), and cornmeal agar (CMA; 8.5 g cornmeal, 1.5% agar). Since the fungi grew well on commonly used MEA compared to the other tested media (data not shown), MEA medium was finally selected for the routine culture and experiments in this study. To evaluate whether silver nanoparticles exert antifungal activity, fungal pathogens, *R. solani*, *S. sclerotiorum*, and *S. minor*, were grown on MEA plates supplemented with different concentrations of silver nanoparticles. A remarked inhibition of hyphal growth and

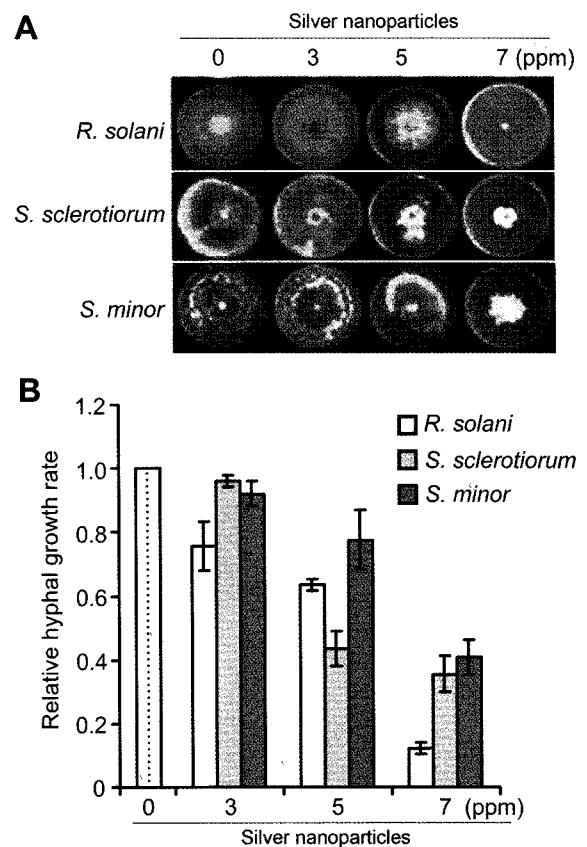


Fig. 1. Effect of silver particles on hyphal growth of *R. solani*, *S. sclerotiorum*, and *S. minor*. **A**, Radial hyphal growth on MEA medium containing the indicated concentrations of silver nanoparticle. Non-treatment served as a control. An agar plug (4 mm in diameter) obtained from the actively growing edge of the fungi was inoculated in the center of plates. Pictures shown were taken at 7 days after inoculation. **B**, Relative hyphal growth rate on MEA medium containing silver nanoparticles. Data were obtained from triplicate assays; data are presented as means±SD.

abnormal patches of aerial hyphal mass were observed in the treatment of silver nanoparticles at concentrations higher than 5 ppm (Fig. 1A and B). Measurement of radial hyphal growth revealed that the silver nanoparticle retarded fungal growth in a dose-dependent manner; value of hyphal growth rate for *R. solani*, *S. sclerotiorum*, and *S. minor* was 12%, 36%, and 41% at 7 ppm of silver nanoparticles-supplemented medium, respectively, compared to the non-treatment (Fig. 1B). Different antimicrobial efficiency of the silver nanoparticles was observed on tested sclerotium-forming fungi. *R. solani* showed the highest growth inhibition in the treatment of silver nanoparticles, followed by *S. sclerotiorum* and *S. minor*.

Effect of silver nanoparticles on the sclerotial germination growth. Effect of silver nanoparticles on the fungal sclerotial germination growth was assayed. Briefly, freshly obtained sclerotia of *R. solani*, *S. sclerotiorum*, and *S. minor* were placed at the center of MEA plates supplemented with either indicated concentrations of the silver nanoparticles or equal amount of water (Fig. 2A and B). The measurement of sclerotial germination growth was conducted when the controls reached the edge of plates.

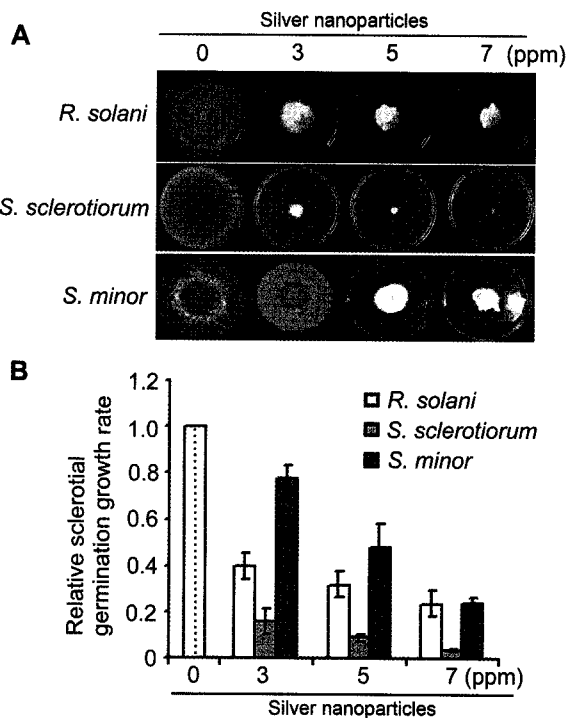


Fig. 2. Effect of silver nanoparticles on sclerotial germination growth of *R. solani*, *S. sclerotiorum*, and *S. minor*. **A**, Sclerotial germination growth on MEA medium containing indicated concentrations of silver nanoparticle. Pictures were taken when controls covered the plates. **B**, Relative sclerotial germination growth rate. Data were obtained from triplicate assays; data are presented as means \pm SD.

Treatments of the silver nanoparticles clearly showed the inhibition of sclerotial germination growth of tested fungal pathogens while actively growing hyphae after the sclerotial germination were obviously observed on water-treated plates (Fig. 2). Their growth phenotypes appeared abnormal on the silver-contained plates, having compact patches of aerial hyphae (Fig. 2A). The silver nanoparticles showed the highest inhibition effect on sclerotial germination growth of *S. sclerotiorum*, compared to that of the other fungi (Fig. 2). Sclerotial germination of *S. sclerotiorum* was almost inhibited at a highest concentration (7 ppm) of the silver nanoparticles tested, while that of *R. solani* and *S. minor* were suppressed (>75%). Continuous growth after sclerotial germination was not formed in a prolonged incubation on plates containing 7 ppm silver nanoparticles (data not shown).

Effect of silver nanoparticles on hyphal growth. As demonstrated above, silver nanoparticles inhibited the hyphal growth and sclerotial germination growth. In order to elucidate the effect of silver nanoparticles on fungal growth, healthy fungal hyphae grown on MEA plates were sprayed with 10 ppm silver nanoparticle solution, and observed under an electron microscope. This microscopic observation revealed that silver nanoparticles clearly damaged hyphae (Fig. 3B to D), while hyphae treated with water appeared to remain intact (Fig. 3A). In the treatment

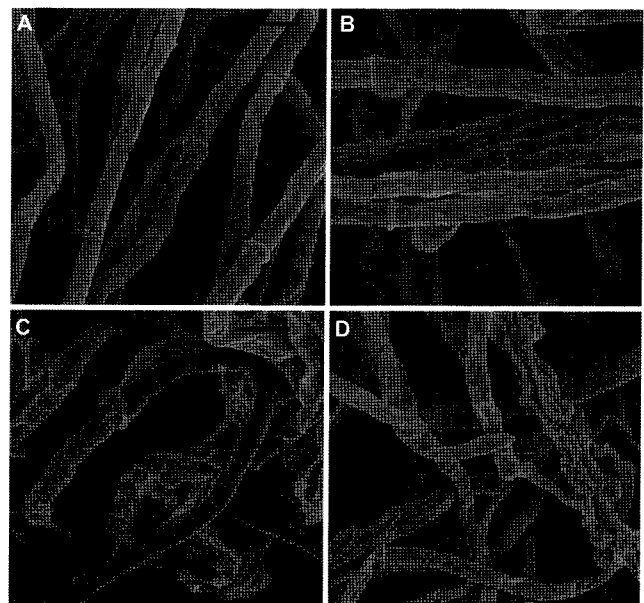


Fig. 3. Electron micrographs of hyphae treated with silver nanoparticles. Fungal hyphae grown on MEA plates were sprayed with either water as a control (A) or equal volume of 10 ppm silver nanoparticle solution (B to D). Photos of B, C, and D were taken 1-, 2-, and 3-day after treatment, respectively. Scale bar=5 μ m.

of silver nanoparticles, the shape of hyphal walls turned abnormal one day, layers of hyphal walls were tore off the damaged hyphae at 3 days, and finally many hyphae were collapsed at 5 days.

Discussion

Little is known about the effect of silvers on phytopathogenic fungi because many studies have focused on bacterial and viral pathogens for animals. Here we evaluated the antifungal activity of silver nanoparticles against sclerotium-forming phytopathogens, *R. solani*, *S. sclerotiorum*, and *S. minor*. Our data clearly demonstrated that the nanoparticles strongly inhibited the fungal growth and sclerotial germination growth. It was suggested that nanometer-sized silvers possess different properties, which might come from morphological, structural and physiological changes (Nel et al., 2003). Indeed, several evidences support enhanced efficiency of silver nanoparticles on antimicrobial activity. Silver nanoparticles are highly reactive as they generate Ag⁺ ions while metallic silver is relatively unreactive (Morones et al., 2005). It was also shown that the nanoparticles efficiently penetrate into microbial cells, which implies lower concentrations of nano-sized silvers would be sufficient for microbial control. This would be efficient, especially for some organisms that are less sensitive to antibiotics due to the poor penetration of some antibiotics into cells (Samuel and Guggenbichler, 2004). A previous study observed that silver nanoparticles disrupt transport systems including ion efflux (Morones et al., 2005). The dysfunction of ion efflux can cause rapid accumulation of silver ions, interrupting cellular processes at their lower concentrations such as metabolism and respiration by reacting with molecules. Also, silver ions are known to produce reactive oxygen species (ROS) via their reaction with oxygen, which are detrimental to cells, causing damage to proteins, lipids, and nucleic acids (Hwang et al., 2008; Storz and Imlay, 1999).

Our microscopic data revealed that silver nanoparticle-treated hyphae were seriously damaged on hyphal walls, resulting in the plasmolysis of hyphae. Considering many cellular effects of silver ions, silver nanoparticle-mediated collapse in *S. sclerotiorum* hyphae is probably not only by the damage on hyphal walls, but also other cellular effects, which need to be characterized. This study suggests the possible use of silver nanoparticles as an alternative to chemical pesticides for the eradication of phytopathogens even though there are some parameters to be evaluated for practical use. These may involve the evaluation of phytotoxicity and antimicrobial effects in hosts, and development of delivery systems of silver nanoparticles into host tissues colonized by phytopathogens.

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