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An *In Vitro* Study of the Antifungal Effect of Silver Nanoparticles on Oak Wilt Pathogen *Raffaelea* sp.

Kim, Sang Woo[1], Kyoung Su Kim[1], Kabir Lamsal[1], Young-Jae Kim[1], Seung Bin Kim[2], Mooyoung Jung[3], Sang-Jun Sim[4], Ha-Sun Kim[4], Seok-Joon Chang[4], Jong Kuk Kim[5], and Youn Su Lee[1]*

[1]*Division of Bio-Resources Technology, Kangwon National University, Chuncheon 200-701, Korea*
[2]*Department of Chemistry, POSTECH, Pohang 790-784, Korea*
[3]*School of Technology Management, Ulsan National Institute of Science and Technology, Ulsan 689-805, Korea*
[4]*Forest Research Institute of Gangwon Province, Chuncheon 200-140, Korea*
[5]*Division of Forest Resources, Kangwon National University, Chuncheon 200-701, Korea*

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In this study, we investigated the antifungal activity of three different forms of silver nanoparticles against the unidentified ambrosia fungus *Raffaelea* sp., which has been responsible for the mortality of a large number of oak trees in Korea. Growth of fungi in the presence of silver nanoparticles was significantly inhibited in a dose-dependent manner. We also assessed the effectiveness of combining the different forms of nanoparticles. Microscopic observation revealed that silver nanoparticles caused detrimental effects not only on fungal hyphae but also on conidial germination.

Keywords: Silver nanoparticles, *Raffaelea*, ambrosia, oak wilt.

Mortality of oaks in Korea has been rapidly increasing since 2004. Tens of thousands of oaks have been killed by an unidentified fungal species of the genus *Raffaelea.* Oak wilt caused by *Raffaelea* sp. has become a major destructive disease in Korea, damaging both forest and landscape oaks. *Raffaelea* sp. is an ambrosia fungus that invades water-conducting tissues of diverse tree species. This invasion leads to cavitation, discoloration, desiccation, and dysfunction of xylem vessels, thereby blocking upward water flow [12]. Extensive disease development in the vessels causes severe wilt of foliage and subsequent death of the host. Ambrosia fungi have a symbiotic relationship with ambrosia beetles. The fungi serve as a nutrient source during insect development in galleries and are dispersed to new habitats primarily *via* the mycangium, a specialized insect organ adapted to the transport of symbiotic fungi [1, 8, 10]. In Korea, *Raffaelea* sp. is predominantly observed in the galleries and mycangia of an ambrosia beetle, *Platypus koryoensis,* in wilted oak trees, suggesting that this fungus is responsible for the oak wilt disease [1, 8, 10]. Similarly, this hypothesis is supported by the results of an inoculation test in which oak wilt disease in Japan was proved to be associated with a related fungus, *R. quercivora* [11]. Identification of the *Raffaelea* species that is causing the death of Korean oaks is a very important task, but it has yet to be accomplished. Approximately 13 species of the genus have been reported, including *R. montetyi, R. ambrosiae,* and *R. tritirachium* [9]. However, the identification and classification of species of *Raffaelea* have remained challenging tasks since the early taxonomic establishment of the genus [6]. This difficulty is due to the lack of distinct differences in morphological characteristics between species. For example, conidia and conidiophores lack pigmentation and other distinguishing features, and conidiogenesis is barely visible.

It is very difficult to control oak wilt because the advanced disease becomes well-established within trees prior to the appearance of wilt symptoms in summer [12]. Few fungicides have been effective in curing diseased trees. Use of the systemic fungicide propiconazole (sold under the trade name Alamo) for oak wilt diseases caused by the fungal pathogen *Ceratocystis fagacearum* is limited owing to high cost. Current control measures are concentrated mostly on preventing dissemination of the disease to uninfected plants; diseased trees are removed and treated with fumigants. Finding or developing a new fungicide that is effective against deadly oak wilt pathogens is a priority for protecting oak species.

*Corresponding author
Phone: +82-33-250-6417; Fax: +82-33-244-6410;
E-mail: younslee@kangwon.ac.kr

Silver has been used in many applications in pure free metal or compound form because it possesses antimicrobial activity against pathogens but is nontoxic to humans [3, 18]. Silver ions are very reactive, leading to inhibition of microbial respiration and metabolism as well as physical damage [2, 17]. Moreover, it has been suggested that silver ions intercalate into bacterial DNA once entering the cell, which prevents further proliferation of the pathogen [4]. Recently, nanotechnology has amplified the effectiveness of silver particles as antimicrobial agents. The larger surface area-to-volume ratio of silver nanoparticles increases their contact with microbes and their abilility to permeate cells. Nanoparticle development has restored interest in the antimicrobial effects of metals, which declined following the widespread application of modern synthetic antibiotics. Unfortunately, studies on the antimicrobial activity of silver nanoparticles have been performed mostly on animal pathogens [2, 3, 4, 15]. In this study, we investigated the effects of three different forms of silver nanoparticles on the ascomycetous phytopathogen *Raffaelea* sp. that causes oak wilt in Korea.

MATERIALS AND METHODS

Fungal Pathogen, Culture Conditions, and Silver Nanoparticles

Raffaelea sp. was routinely grown on several media, including 2% malt agar (MA, 0.2% [w/v] malt extract, 1.5% [w/v] agar) medium at room temperature. For measurement of hyphal growth, an agar plug (4 mm in diameter) was obtained from the actively growing edge of the fungus, inoculated into the center of a culture plate containing MA medium, and incubated for 7 days at 24°C. Silver nanoparticles (Nanover) were obtained from BioPlus Co., Ltd. (Korea). The three different forms of Nanover, CV-WA13 (CV), AT-WB13R (AT), and PR-WB13 (PR), all dissolved in distilled water, were utilized in this study. These nanoparticles have an average size of 4–8 nm.

Conidial Germination

Conidia were obtained from hyphal mats of *Raffaelea* sp. grown for 2 weeks on MA medium. Ten ml of sterile distilled water was added to culture plates, and then conidia were harvested with a glass rod and filtered through Miracloth (Calbiochem, La Jolla, CA, U.S.A.). After adjusting the concentration of conidia to 10^4/ml, 1 ml of the suspension was spread on MA plates supplemented with either 10 ppm AT or an equal volume of water. The plates were incubated for 2 days at 24°C and used to observe conidial germination.

Scanning Electron Microscopy (SEM)

A culture of *Raffaelea* sp. grown on MA medium plates was sprayed with 5 ml of AT solution (10 ppm) and incubated for 3 days. 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 under a Hitachi S-3500N scanning electron microscope at an accelerating voltage of 10 kV.

RESULTS

Effect of Silver Nanoparticles on Growth of *Raffaelea* sp.

First, we compared the growth of *Raffaelea* sp. on several synthetic media, including MA, potato dextrose agar (PDA), DYPA (20 g dextrose, 5 g yeast extract, 2 g peptone, 1.5% agar), and OA (17 g oatmeal, 1.5% agar). Since the fungus grew very slowly on commonly used PDA compared with the other media tested (data not shown), MA medium was finally selected for routine culture. To evaluate whether silver

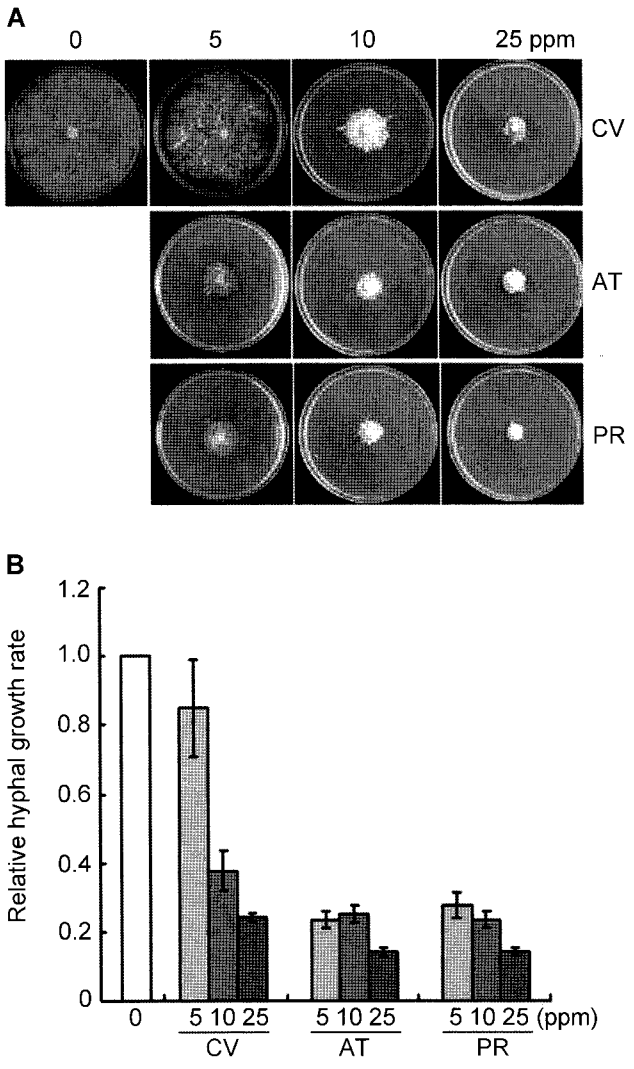

Fig. 1. Effect of silver nanoparticles on hyphal growth in *Raffaelea* sp.

A. Radial hyphal growth on MA medium containing the indicated concentrations of each form of silver nanoparticles. Non-treatment served as a control (top left panel). An agar plug (4 mm in diameter) obtained from the actively growing edge of the wild-type strain was inoculated in the center of the plates. Pictures shown were taken at 7 days post-inoculation. Three independent experiments were performed. **B.** Relative hyphal growth rate on MA medium containing silver nanoparticles. Colony diameters were measured at 7 days post-inoculation. Data were obtained from triplicate assays; data are presented as means±SD.

nanoparticles possess antifungal activity against *Raffaelea* sp., the fungus was grown on MA plates supplemented with various concentrations of each different Nanover form. Significant inhibition of hyphal growth and abnormal patches of aerial hyphal mass were observed following treatment with CV, AT, or PR at concentrations higher than 10 ppm (Figs. 1A and 1B). Measurement of radial hyphal growth revealed that each type of silver nanoparticles retarded fungal growth in a dose-dependent manner; the hyphal growth rate was 0.24, 0.12, or 0.12 at 25 ppm CV, AT, or PR, respectively, relative to the value of 1 corresponding to non-treatment (Fig. 1B). Differences in antimicrobial efficiency among the different forms of silver nanoparticles were also observed. The inhibition efficiency of CV overall was less than that of AT and PR. For example, CV did not inhibit fungal growth at 5 ppm, whereas AT and PR were effective at this concentration.

Effect of Combining Silver Nanparticles on Fungal Growth

Since there was a difference in antifungal activity between the three forms of silver nanoparticles, we investigated the effect of combining the different forms on fungal growth. As shown in Fig. 2, when CV was combined with either AT or RP, increased inhibition of fungal growth was clearly observed (Fig. 2A), compared with treatment with CV alone. In particular, we observed relative fungal growth decreases from 0.85 to 0.27, from 0.38 to 0.25, and from 0.24 to 0.15 when CV was combined with AT using 5, 10, and 25 ppm nanoparticles, respectively (Figs. 1B and 2B). However, the synergistic effect of combining CV with AT and/or PR was less than the effect of combining AT and PR; the latter combination showed the strongest antifungal activity. This result suggests that CV may interfere to some extent with microbial absorption.

Effect of Silver Nanoparticles on Hyphae of *Raffaelea* sp.

As described above, silver nanoparticles inhibited the growth of fungal hyphae. In order to visualize the microscopic effect of this treatment, healthy fungal hyphae grown on MA plates were sprayed with 10 ppm AT solution, and then observed under an electron microscope. Breakage of hyphal tips, where new conidia form, as well as detached conidia, were detected simultaneously (Fig. 3). Damage to the surface of the fungal hyphae was also observed, which could have caused the release of internal cellular materials, resulting in shrinkage of the hyphae. Contrary to this observation, hyphae treated with water appear to have remained intact (Fig. 3).

Inhibition of Conidial Germination

The effect of silver nanoparticles on conidial germination in *Raffaelea* sp. was assayed on Petri plates. Microscopic observation revealed that conidial germination was inhibited on plates containing silver nanoparticles, whereas actively growing hyphae resulting from conidial germination were clearly observed on plates treated with water only (Fig. 4). Germination did not occur following prolonged incubation of conidia treated with nanosilver (data not shown).

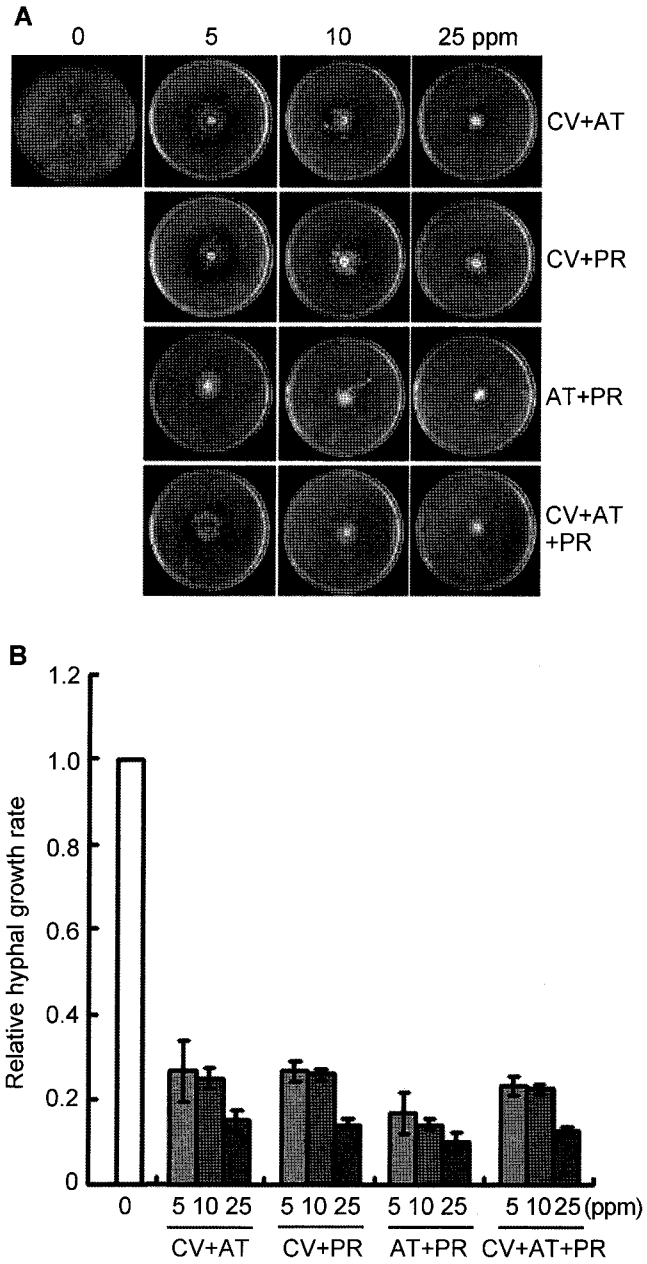

Fig. 2. Effect of combining different forms of silver nanoparticles on hyphal growth in *Raffaelea* sp.
A. Radial hyphal growth on MA medium containing indicated concentrations of silver nanoparticle combinations. Pictures shown were taken at 7 days post-inoculation. Three independent experiments were performed. **B.** Relative hyphal growth rate on MA medium containing indicated concentrations of silver nanoparticle combinations. Colony diameters were measured at 7 days post-inoculation. Data were obtained from triplicate assays; data are presented as means±SD.

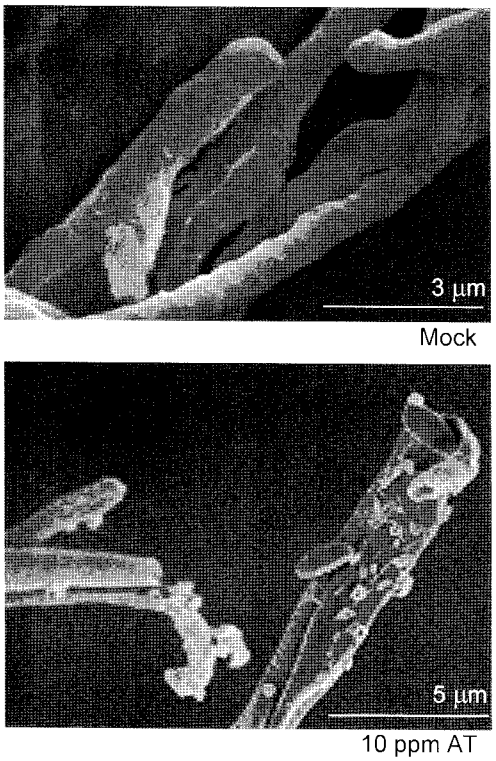

Fig. 3. Electron micrographs of hyphae of *Raffaelea* sp.
Fungal hyphae grown on MA plates were treated with either 10 ppm silver nanoparticle solution AT or an equal volume of water as a control (Mock). Representative photos were taken under SEM 72 h after treatment.

DISCUSSION

Recently, several microorganisms have threatened to cause an ecological disaster due to the destruction of a variety of tree species in many countries. Among them, ascomycetous fungi belonging to the genus *Raffaelea* have become major pathogens of trees, causing severe wilt disease. Along with the outbreak of oak death caused by an unidentified *Raffaelea* species in Korea, *R. lauricola* has caused the death of nearly all redbay and sassafras trees in the U.S.A. since 2002 [5]. A symbiotic relationship between ambrosia pathogens and their vectors is responsible for establishing disease in diverse hosts. The outbreak of disease in redbay trees is associated with a newly introduced ambrosia beetle, *Xyleborus glabratus* [5]. In Korea, the oak wilt pathogen *Raffaelea* sp. is mainly vectored by the beetle *P. koryoensis*, whereas in Japan, *R. quercivora* is transferred by *P. quercivorus* to oak species that are different from those in Korea [11]. The rapid, potentially catastrophic devastation that has occurred is considered to have resulted from both the loss of biodiversity among tree species and the introduction of foreign pathogens [5]. This dual threat increases the likelihood that almost all oak trees could be destroyed by pathogenic events. The wilt disease caused by *Raffaelea* involves the dysfunction of sapwood [12]. In response to a vector-mediated attack by the pathogen, the host produces secondary metabolites that subsequently cause cavitation or discoloration in the water-conducting system. However, the host response is insufficient to kill the pathogen or prevent its spread. Continued progression of the fungal infection augments xylem dysfunction, causing the death of the host. Unfortunately, there is no known cure or means of controlling the disease.

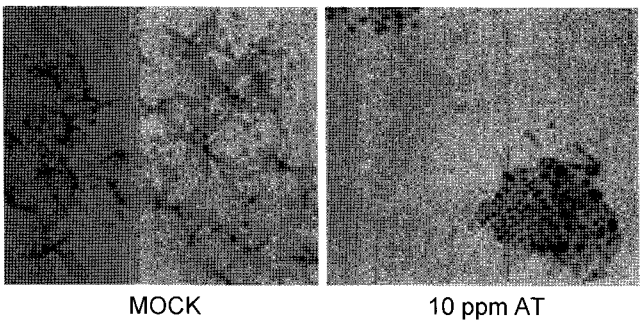

Fig. 4. Effect of silver nanoparticle solution AT on the germination of conidia in *Raffaelea* sp.
Conidia suspension (10^4/ml) was spread on MA medium supplemented with either water (Mock) or 10 ppm AT. Photos were taken under a light microscope 48 h after conidium inoculation.

It has been suggested that nanometer-sized silver particles possess different physical and chemical properties from their macroscale counterparts that alter their interaction with biological structures and physiological processes [14]. Indeed, several pieces of evidence support the hypothesis that silver nanoparticles have enhanced antimicrobial activity. Silver nanoparticles are highly reactive because they generate Ag^+ ions, whereas metallic silver is relatively unreactive [13]. It has also been shown that nanoparticles efficiently penetrate microbial cells, suggesting that lower concentrations of nanosized silver particles would be sufficient for microbial control. This approach could be more efficient than existing treatments, especially for certain organisms that are less sensitive to antibiotics because of their resistance to cell penetration [15]. In a previous study, it was observed that silver nanoparticles disrupt transport systems, including ion efflux [13]. Dysfunction in ion efflux can cause rapid accumulation of silver ions, interrupting cellular processes such as metabolism and respiration by reacting with certain molecules. Moreover, silver ions are known to produce reactive oxygen species (ROS) that are detrimental to cells, causing damage to proteins, lipids, and nucleic acids [7, 16].

Little is known regarding the effects of silver on phytopathogenic fungi, because most studies have focused on bacterial and viral pathogens in animals. Here, we evaluated the antifungal activity of silver nanoparticles against the fungal phytopathogen *Raffaelea* sp. Our data clearly demonstrated that the nanoparticles strongly inhibited fungal growth and development and damaged cell walls. These results suggest the possibility of using

silver nanoparticles to eradicate phytopathogens. Several parameters will require evaluation prior to practical application, including phytotoxicity and antimicrobial effects *in situ*, and development of systems for delivering particles into host tissues that have been colonized by phytopathogens.

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References

1. Batra, L. R. 1963. Ecology of ambrosia fungi and their dissemination by beetles. *Trans. Kansas Acad. Sci.* **66:** 213–236.
2. Bragg, P. D. and D. J. Rannie. 1974. The effect of silver ions on the respiratory chain of *Escherichia coli*. *Can. J. Microbiol.* **20:** 883–889.
3. Elchiguerra, J. L., J. L. Burt, J. R. Morones, A. Camacho-Bragado, X. Gao, H. H. Lara, and M. J. Yacaman. 2005. Interaction of silver nanoparticles with HIV-1. *J. Nanobiotechnol.* **3:** 6.
4. Feng, Q. L., J. Wu, G. O. Chen, F. Z. Cui, T. N. Kim, and J. O. Kim. 2000. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *J. Biomed. Mater. Res.* **52:** 662–668.
5. Fraedrich, S. W. 2008. California laurel is susceptible to laurel wilt caused by *Raffaelea lauricola*. *Plant Disease* **92:** 1469.
6. Gebhardt, H. and F. Oberwinkler. 2005. Conidial development in selected ambrosial species of the genus *Raffaelea*. *Antonie van Leewenhoek* **88:** 61–66.
7. Hwang, E. T., J. H. Lee, Y. J. Chae, Y. S. Kim, B. C. Kim, B. I. Sang, and M. B. Gu. 2008. Analysis of the toxic mode of action of silver nanoparticles using stress-specific bioluminescent bacteria. *Small* **4:** 746–750.
8. Ito, S., T. Kubono, N. Sahashi, and T. Yamada. 1998. Associated fungi with the mass mortality of oak trees. *J. Japan For. Soc.* **80:** 170–175.
9. Jones, G. J. and M. Blackwell. 1998. Phylogenetic analysis of ambrosial species in the genus *Raffaelea* based on 18S rDNA sequences. *Mycol. Res.* **102:** 661–665.
10. Kinuura, H. 2002. Relative dominance of the model fungus, *Raffaelea* sp., in the mycangium and proventriculus in relation to adult stages of the oak platypodid beetle, *Platypus quercivorus* (Coleoptera; Platypodidae). *J. For. Res.* **7:** 7–12.
11. Kinuura, H. and M. Kobayashi. 2006. Death of *Quercus crispula* by inoculation with adult *Platypus quercivorus* (Coleoptera: Platypodidae). *Appl. Entomol. Zool.* **41:** 123–128.
12. Kuroda, K. 2001. Response of *Quercus* sapwood to infection with the pathogenic fungus of a new wilt disease vectored by the ambrosia beetle *Platypus quercivorus*. *J. Wood Sci.* **47:** 425–429.
13. Morones, J. R., J. L. Elechiguerra, A. Camacho, K. Holt, J. B. Kouri, J. T. Ramirez, and M. J. Yacaman. 2005. The bactericidal effect of silver nanoparticles. *Nanobiotechnology.* **16:** 2346–2353.
14. Nel, A., T. Xia, L. Mädler, and N. Li. 2003. Toxic potential of materials at the nanolevel. *Science* **311:** 622–627.
15. Samuel, U. and J. P. Guggenbichler. 2004. Prevention of catheter-related infections: The potential of a new nano-silver impregnated catheter. *Int. J. Antimicrob. Agents* **23S1:** S75–S78.
16. Storz, G. and J. A. Imlay. 1999. Oxidative stress. *Curr. Opin. Microbiol.* **2:** 188–194.
17. Thurman, K. G. and C. H. P. Gerba. 1989. The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses. *Crit. Rev. Environ. Control* **18:** 295–315.
18. Yeo, S. Y., H. J. Lee, and S. H. Jeong. 2003. Preparation of nanocomposite fibers for permanent antibacterial effect. *J. Mater. Sci.* **38:** 2143–2147.