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# Use of silver nanoparticles for managing *Gibberella fujikuroi* on rice seedlings

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ABSTRACT

It is of great interest for rice producers to develop novel seed disinfection methods with effective, less toxic, and environment-friendly active ingredients. This study evaluated silver nanoparticles (AgNP) as an alternative agent for seed treatment since elemental silver possesses strong antimicrobial properties and a low toxicity to humans and animals. AgNP (~7.5 nm in diameter) solution was chemically synthesized and evaluated for antifungal activity against *Gibberella fujikuroi* as representing a seedborne fungal pathogen in rice (*Oryza sativa*). AgNP reduced viability of *G. fujikuroi* conidia by 50% when directly exposed to concentrations ranging from 0.015 to 1.5 μg $mL^{-1}$ for 1 to 20 min. Rice seeds infested with *G. fujikuroi* and treated with 150 μg $mL^{-1}$ of AgNP significantly decreased colony-forming units (CFU) of *G. fujikuroi* on the seed surface when treated for 10 min and up to 24 h. Seed treatment with 150 μg $mL^{-1}$ AgNP for 12 or 24 h significantly improved seedling emergence and height of *G. fujikuroi*-infested seeds. Adverse effects on germination rate and seedling growth were not observed with any of the AgNP treatments (≤150 μg $mL^{-1}$ for up to 48-h exposure) tested in this study. The antifungal effect of AgNP against *G. fujikuroi* attested in this study suggests that AgNP is a new antifungal ingredient that can be used for managing important seedborne fungal pathogens of rice.



## 1. Introduction

Seedborne diseases of rice (*Oryza sativa* L.) cause considerable yield losses in rice-growing regions worldwide. Bakanae disease, caused by *Gibberella fujikuroi* Nirenberg, is one of the most important seedborne fungal diseases, particularly in Asian countries where rice seedlings are prepared in seed boxes for mechanical transplanting to the field (Ou, 1987). *G. fujikuroi* reduces seed germination and slows seedling growth, but does not cause immediate seedling death. Later stages of rice growth are also affected and include symptoms such as elongated seedling leaves, slender stems, and chlorotic leaves due to excessive gibberellin secreted by the pathogen. Furthermore, infected plants that reach maturity fail to produce normal grains. In addition to causing yield loss, some isolates of *G. fujikuroi* produce mycotoxins in rice grains that are harmful to human and animal health (Desjardins et al., 1997).

The biggest challenge in managing this seedborne disease is the lack of effective management practices once disease symptoms are observed. Therefore, the most effective and economically feasible approach to managing the seedborne disease is through preemptive seed treatment. Conventional seed treatments for rice include fungicide, hot water, or chlorine treatment. Fungicides are effective for control of only certain fungal pathogens (Fernandez et al., 2009; Wise et al., 2009). Since all fungicides pose a certain level of risk to human and animal health, fungicide handlers must exercise care and follow proper safety procedures to avoid overexposure to chemicals or accidental spills. Fungicide-treated seeds should be handled carefully and regulated so that they are not used for human consumption or livestock feed (U.S. Food and Drug Administration, 2013).

Hot water or chlorine treatments are often used to manage seedborne pathogens as an alternative to fungicides (Du Toit and Hernandez-Perez, 2005; Jaquette et al., 1996). Hot water or chlorine treatments must be carried out accurately and according to standard protocols to achieve disease control and avoid damage to

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seeds because deviations too far from the effective treatment conditions render seeds with poor disinfection or cause seed damage (Du Toit and Hernandez-Perez, 2005). Chlorine treatment is particularly difficult to apply in combination with conventional fungicides as tank mixtures due to incompatibility issues that compromise fungicide active ingredients (Hwang et al., 2001). Therefore, developing efficacious and prophylactic seed disinfection methods using safe active ingredients is an important research area.

Elemental silver has been shown to have minimal toxicity to humans and strong antimicrobial activity (Reddish, 1954). Recent advancements in nanotechnology have enhanced the antimicrobial properties of silver. Silver nanoparticles (AgNP) have shown more effective antimicrobial activity due to enhanced surface area that leads to an increased chance of direct contact with target microbes. Moreover, AgNP has been extensively adapted for use in medical and pharmaceutical products (Dowsett, 2004; Hotta et al., 1998; Matsuura et al., 1997), sterilization processes (Lin et al., 2002; Liu et al., 1994), and textile products (Takai et al., 2002). AgNP has also been shown to manage plant pathogens without causing adverse effects on plants (Jo et al., 2009; Park et al., 2006). The objective of this study is to evaluate the efficacy of chemically synthesized AgNP in reducing a rice seedborne fungal pathogen, *G. fujikuroi*.

## 2. Materials and methods

### 2.1. Synthesis of silver nanoparticles

Silver nitrate ($AgNO_3$) was used as the AgNP source; sodium borohydride ($NaBH_4$) was used as a reducing agent; and starch was used as a stabilizing agent. A 150 μg $mL^{-1}$ solution of AgNP was synthesized via a redox reaction of a silver element in the starch solution by following a previously developed procedure (Fan et al., 2009). A 0.2% (w/v) starch solution was made by completely dissolving 0.4 g of soluble starch in 200 mL of sterilized double-distilled water ($ddH_2O$). The solution was allowed to cool down to room temperature (~25 °C) and was stirred continuously throughout the synthesis procedure. A 3 mL solution containing 0.05 g of $AgNO_3$ was added to the starch solution and was allowed to mix thoroughly. A 6 mL solution containing 0.02 g of $NaBH_4$ was added with continued stirring to the starch solution. After mixing for an additional 30 min, the resulting AgNP solution was stored in a refrigerator at 4 °C until use.

### 2.2. Characterization of silver nanoparticles

Ultraviolet (UV)–visible spectra of AgNP synthesized in this study were produced using a UV–Vis–NIR spectrophotometer (U-4100, Hitachi High Technologies America, Inc., Dallas, TX) for measuring AgNP sizes. The AgNP solution was diluted to 3% by volume with $ddH_2O$ and placed in a cuvette for absorption spectra measurement.

High-resolution electron micrographs of AgNP were taken using a field-emission transmission electron microscope (FE-TEM, FEI Tecnai G2 F20) operating at 200 KeV. AgNP were dispersed in ethanol and drop-casted on a carbon-coated, 300-mesh copper grid. The grid was then air-dried under ambient conditions before microscopic examination.

### 2.3. Direct exposure of silver nanoparticles to G. fujikuroi conidia

The antifungal activity of AgNP was evaluated by mixing a conidial suspension of *G. fujikuroi* directly with AgNP solution. *G. fujikuroi* isolate FGSC #8381 (McCluskey, 2003) was used as a representative isolate. The fungus was grown on V8 juice agar medium [20% (v/v) V8 vegetable juice, 3 g $CaCO_3$, 2% (w/v) agar per liter] at 25 °C for 2 wk to allow mycelium to cover the surface of the medium and sporulate. Conidia were harvested and diluted with sterile $ddH_2O$ to yield a concentration of $7 \times 10^6$ $mL^{-1}$.

The *G. fujikuroi* conidia suspension was mixed with AgNP solution in a 1:99 ratio. This made the final conidia concentration $7 \times 10^4$ $mL^{-1}$. After incubation (25 °C) with designated exposure times, an aliquot of 100 μL of treated conidia was taken and serially diluted with sterile $ddH_2O$ to an appropriate concentration to achieve countable colony-forming units (CFU) in one petri dish (100 mm in diameter, 15 mm deep). The aliquot was spread on potato dextrose agar medium (PDA) amended with ampicillin (75 μg $mL^{-1}$). Conidia mixed with sterile $ddH_2O$ were included and served as the non-treated control. Plates were incubated at 25 °C for 3 d, and CFU on each plate was counted. Three plates (replications) were used for each treatment.

To estimate AgNP doses for killing *G. fujikuroi*, conidial suspensions were treated with eight concentrations of AgNP (0, 0.00015, 0.0015, 0.015, 0.15, 1.5, 15, or 150 μg $mL^{-1}$), and then the mixtures of conidia and AgNP were incubated at 25 °C for 1, 10, or 20 min. CFU for each AgNP dose and exposure time were counted. The lethal concentrations of AgNP inhibiting 50% CFU ($LC_{50}$) of *G. fujikuroi* were estimated.

### 2.4. Seed surface disinfection with silver nanoparticles

Rice seeds (cultivar Cocodrie) were washed by vortexing at 3200 rpm in sterile $ddH_2O$ for 3 min. During each washing run, 100 rice seeds were added to a 250 mL Erlenmeyer flask. The washing step was continued with fresh sterile $ddH_2O$ for three times. Washed seeds were air-dried on a clean bench for 1 h. The seeds were soaked in a conidia suspension ($5 \times 10^5$ $mL^{-1}$) at 25 °C for 24 h to allow conidia to attach to the seed surface, and the infested seeds were air-dried on a clean bench for 1 h.

Thirty-five infested seeds were completely submerged in 150 μg $mL^{-1}$AgNP solution and 35 infested seeds in sterile $ddH_2O$ as the non-treated water control. Subsequently, five seeds were recovered after the following exposure durations: 1/6, 1/3, 1/2, 1, 3, 6, or 24 h. Five infested seeds without submersion in AgNP solution or $ddH_2O$ were included and used for measuring the initial CFU before the treatments. Each recovered seed was placed in a sterile microcentrifuge tube containing 500 μL of sterile $ddH_2O$ and shaken using a Mini Beadbeater (Biospec Products, Inc., Bartlesville, OK) at 1500 rpm for 3 min. An aliquot (100 μL) of the treated conidia solution was taken and serially diluted with sterile $ddH_2O$ to an appropriate concentration to achieve countable CFU on PDA amended with ampicillin after incubation at 25 °C for 3 d. Fungal colonies other than *G. fujikuroi* in the same plate were excluded from counting. Morphological characteristics used for *G. fujikuroi* identification were rosy-white mycelium and production of mirco- and macro-conidia (Ou, 1987), and were compared with *G. fujikuroi* original reference cultures.

### 2.5. Effect of G. fujikuroi on seedling emergence and height with or without silver nanoparticles

Seeds infested with *G. fujikuroi* were prepared using the aforementioned method. The infested seeds were treated with AgNP or sterile $ddH_2O$ as the non-treated control (positive control) for 12 or 24 h. The treated seeds were briefly air-dried on sterile filter paper and planted 2 cm deep in round plastic pots (10 cm in diameter, 8.5 cm deep) containing 45 g of soil mixture (Metro-Mix 200, Sun Gro Horticulture, Bellevue, WA). Non-infested and non-treated seeds were also included as the negative control. Five seeds were