

Article

# Silver Nanoparticle-Based Paper Packaging to Combat Black Anther Disease in Orchid Flowers

Bang-on Nokkrut, Sawitree Pisuttipiched, Somwang Khantayanuwong and Buapan Puangsin \* 

Department of Forest Products, Faculty of Forestry, Kasetsart University, Bangkok 10900, Thailand; Puziz\_Model@hotmail.co.th (B.N.); fforsap@ku.ac.th (S.P.); fforsok@ku.ac.th (S.K.)

\* Correspondence: fforbpb@ku.ac.th; Tel.: +66-2942-8109 (ext. 1918)

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**Abstract:** Metal nanoparticles have been reported to have a high antimicrobial activity against fungi, bacteria, and yeasts. In this study, we aimed to synthesize silver nanoparticles (AgNPs) using a chemical reduction method at 90 °C. The obtained AgNPs were used as an antifungal coating on packaging paper, to control the growth of *Colletotrichum gloeosporioides* in cut orchid flowers during the shipping process. The AgNPs were characterized by a UV-Vis spectroscopy and atomic force microscope (AFM). The results indicated that their shape was spherical and homogenous, with an average size of 47 nm. An AgNPs concentration of 20 and 50 particles per million (ppm), mixed with starch, was prepared as the coating solution. The paper coated with a concentration of 50 ppm exhibited a significant antifungal activity against *C. gloeosporioides* compared to 20 ppm. The coated paper had a higher water resistance and better mechanical properties compared to the uncoated paper. Additionally, we observed a significant reduction in the number of orchid inflorescence anthers, infected by *C. gloeosporioides*, when stored in the coated boxes. The current study demonstrates that paper boxes, coated with AgNPs, can be used in controlling the *C. gloeosporioides* infection during storage of cut orchid flowers.

**Keywords:** black anther disease; orchid cut flower; silver nanoparticles

## 1. Introduction

The orchid is one of the most important commercially viable ornamental plants in Thailand, especially the cut flowers and potted plants [1,2]. Around 1300 species and 180–190 orchid genera have been reported, that are grown widely across the country [3]. Thailand is one of the world's largest exporters of cut orchid flowers and has had a long history of orchid trading around the world [2]. The total orchid flower export business is valued at around 59–70 million US\$ (approximately 1949–2307 million Thai Baht) [2]. In addition, the exports of cut orchid flowers experienced a decrease of about 24.2 million tons, valued at approximately 67.5 million US\$ (approximately 2228 million Thai Baht), in 2017. Black anther, caused by the phytopathogen *Colletotrichum gloeosporioides*, causes a significant reduction in the postharvest quality of cut orchid flowers, especially during the rainy season [3,4]. The symptoms of this disease include a black spot on the anther of the flower [5], lowering the quality and a shorter vase life, and in turn, a reduction in the export value.

Synthesized fungicides, such as thiabendazole, prochloraz, azoxystrobin, and chlorothalonil, have been commonly used to control the growth of *C. gloeosporioides* in the orchid, during postharvest shelf life; however, their excessive use has resulted in the fungi becoming increasingly resistant to the fungicides. At the same time, there has been an increasing concern related to consumer safety [5,6]. The development of an antimicrobial packaging paper, with properties that can prolong the shelf life of the product during storage or transportation, while maintaining an acceptable quality, has been gaining the attention of researchers [7,8]. Different types of antimicrobial agents, such as silver nanoparticles

(AgNPs) [6,9,10], zinc pyrithione [11], benzimidazole [12], organic acids [13], borate [14], and plant extraction [15], have been reported to be potential coating material in paper boxes, in order to control the growth of *C. gloeosporioides*. However, organic and natural biological antimicrobials have been reported to be less stable at higher temperatures and have high volatility, compared to inorganic ones [16,17], which may result in a limited application.

To our knowledge, studies related to the use of antimicrobial coating on paper boxes for the inhibition of *C. gloeosporioides* in harvested orchid flower packaging in Thailand have yet to be made. Therefore, the main objective of this study was to synthesize AgNPs by a chemical reduction method and use it as an antifungal agent. The packaging paper was coated with an appropriate amount of AgNPs, mixed with a starch solution, to increase its antifungal properties. The morphology, basis weight, thickness, mechanical properties, and water resistance of the paper were evaluated. Additionally, the antifungal activity of coated paper, to combat *C. gloeosporioides*, by disc diffusion, was evaluated. Finally, the efficacy of the antifungal coating, in inhibiting the proliferation of black anther disease in stored cut orchid flowers, was also investigated.

## 2. Materials and Methods

### 2.1. Materials

Uncoated paper (134 g/m<sup>2</sup>), commonly used for the storage of orchid flowers, was obtained from Mahachai Kraft Paper Co. Ltd. (Samutsakorn, Thailand). Hydrophobic starch (FILMKOTE 370TM) was supplied by National Starch and Chemical (Thailand) Co. Ltd. (Samutprakan, Thailand). Chemicals used to prepare AgNPs, i.e., Silver nitrate (AgNO<sub>3</sub>) and Sodium hydroxide (NaOH), were purchased from Merck Co. (Darmstadt, Germany). Trisodium citrate (Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>) and sodium borohydride (NaBH<sub>4</sub>) were purchased from Ajax Finechem Co. (Victoria, Australia). Potato dextrose agar (PDA) was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). *C. gloeosporioides* was obtained from the Department of Agriculture, Ministry of Agriculture and Cooperatives (Bangkok, Thailand). The cut orchid flowers (*Dendrobium sonia*) were collected from the Siamtaiyoo farm Co. Ltd. (Samut Sakhon, Thailand).

### 2.2. Synthesis of Silver Nanoparticles

AgNPs were prepared by the chemical synthesis method outlined in Agnihotri et al. [18]. Briefly, a mixture of aqueous solution containing 24 mL of sodium borohydride ( $2 \times 10^{-3}$  mol·dm<sup>-3</sup>) and 24 mL of trisodium citrate ( $4.28 \times 10^{-3}$  mol·dm<sup>-3</sup>) was heated to 60 °C for 30 min, in the absence of light. Silver nitrate ( $1 \times 10^{-3}$  mol·dm<sup>-3</sup>) solution (2 mL) was then added to the mixture, and heated from 60 to 90 °C. The reaction was allowed to continue for an additional 20 min and then cooled down to room temperature. The UV-visible spectrum properties of the synthesized AgNPs solution were determined, using a spectrophotometer (UV-1800, Shimadzu Corp., Kyoto, Japan), at wavelengths ranging between 300–700 nm. The dimensions of AgNPs were examined through an atomic force microscope (MFP-3D (Bio), Asylum Research Corp., Santa Barbara, CA, USA). A drop of AgNP solution was placed on a glass plate and dried in a desiccator for 24 h, prior to an analysis in the atomic force microscope (AFM), set on the tapping mode and a scan rate of 0.80 Hz.

### 2.3. Preparation of Antifungal Coating Solution

The antifungal coating solution was prepared using 8 g of hydrophobic starch, added to 100 mL of deionized water. The mixture was then heated and stirred at  $90 \pm 3$  °C for 30 min. The obtained starch solution was cooled down to  $65 \pm 3$  °C and the AgNP solution added to the predetermined quantity (0, 20, and 50 ppm) before use.

#### 2.4. Preparations of Antifungal Coating Papers

The blended solution was coated on multiple papers (180 × 180 mm) using the bar coating method. The coated papers were then dried in an oven at  $105 \pm 2$  °C for 15 min. All the coated papers had a constant coated weight of  $4 \pm 0.5$  g/m<sup>2</sup>. The morphology of both the surface and the cross-section of the paper samples were examined through a field emission scanning electron microscope (FE-SEM) (Su8020, Hitachi, Tokyo, Japan), at an accelerating voltage of 5 kV. The paper samples were sputtered with a platinum coating of 10 nm thickness.

#### 2.5. Antifungal Activity of the Coated Paper

The antifungal activity of the coated paper, against a fungal stain of *C. gloeosporioides*, was evaluated using the disc diffusion method. Briefly, a PDA disc of diameter 6 mm, containing *C. gloeosporioides*, was placed on the surface of a PDA plate (90 mm diameter) using a sterile cork borer. The coated paper discs, of diameter 6 mm, were placed at the center of the PDA medium. This plate was incubated at 25 °C for 7 days and the growth diameter was measured 3, 5, and 7 days after the incubation, with the experiment being repeated five times. The percentage inhibition was calculated using the equation:

$$\text{Inhibition (\%)} = (A - B/A) \times 100 \quad (1)$$

where *A* is the fungal colony radius of the control plate containing the PDA without the paper sample and *B* is the colony radius in the test plate containing the PDA and paper sample.

#### 2.6. Basis Weight and Thickness

Basis weight and thickness of the uncoated and coated papers was measured according to ISO 536:2012 [19] and ISO 534:2011 standards [20], respectively. The weight of 10 individual papers was measured and the mean values were calculated. The thickness of the papers was measured using a micrometer (Lorentzen & Wettres, Stockholm, Sweden). Each paper was randomly measured at five different places and the mean thickness of a single paper was calculated.

#### 2.7. Tensile and Bursting Test

A universal testing machine (Vantage NX, Thwing-Albert Instrument Co. Ltd., Philadelphia, PA, USA) was used to test the tensile strength, according to the ISO 1924-2:2008 standard [21]. The gauge length was 10 cm and the crosshead speed set at 50 mm/min. The papers were cut to a width of  $15 \pm 0.1$  mm and length of  $180 \pm 1$  mm. For the bursting strength was tested using a burst test machine (MTA-2000, Regmed Indústria Técnica de Precisão Ltda., Osasco, Brazil), according to the ISO 2758:2001 standard [22]. The measurement was done on 10 replicates of sample papers.

#### 2.8. Water Absorptiveness

The water absorptiveness of the uncoated and coated papers was determined using the Cobb method according to the ISO 535:1991 standard [23]. The papers were cut into squares of size  $14 \times 14$  cm<sup>2</sup> and clamped inside the ring of a Cobb tester, having an area of 100 m<sup>2</sup>. Into the ring, 100 mL of distilled water was poured the water was absorbed for 120 s. The excess water was then poured out and a wet paper was placed between the blotting papers in order to remove the excess surface water on the paper. The Cobb value was measured in terms of the amount of distilled water in g/m<sup>2</sup> and the experiment was repeated 10 times.

#### 2.9. Antifungal Activity of the Coated Paper

The cut orchid flowers (*Dendrobium sonia*) were collected from Siamtaiyoo farm Co. Ltd. (Samut Sakhon, Thailand) to test the antifungal efficacy of the paper. Healthy flowers were selected based on a long stem (45 cm), flower with approximately  $7 \pm 1$  blooms, and  $5 \pm 1$  buds per stem (export quality

grade). Boxes of dimensions 52 cm × 40 cm × 60 cm (height × width × length), generally used for shipping the flowers, size of, were obtained from Siamtaiyoo farm Co. Ltd. (Samut Sakhon, Thailand). AgNPs coated sample papers were placed in the entire inside of the boxes, using a double sided tape. Forty stems of freshly cut orchid flowers were prepared for the packaging test, and placed in boxes with and without AgNPs coating. From each box, one flower bloom was selected and its anther was wounded by puncturing with a sterilized pin. Twenty µL of spore suspension (106 spore/mL) was then dropped into the wound. The sample boxes were stored at a room temperature of  $25 \pm 2$  °C and 50% RH for 7 days. Table 1 shows the treatments used during the packaging test, with the experiment repeated five times. The percentage infection was calculated from the equation:

$$\text{Infection (\%)} = (B/A) \times 100 \quad (2)$$

where *A* is all the orchid flower blooms and *B* is the number of orchid flower blooms infected with the fungi.

**Table 1.** The treatments used during the packaging test.

Treatment	Packaging	Pulsing Solution
T <sub>0</sub>	Uncoated	Distilled water
T <sub>1</sub>	Uncoated	8-HQS 225 ppm + AgNO <sub>3</sub> 30 ppm + Sucrose 4%
T <sub>2</sub>	Uncoated	8-HQS 225 ppm + AgNPs 20 ppm + Sucrose 4%
T <sub>3</sub>	AgNPs coated	8-HQS 225 ppm + AgNO <sub>3</sub> 30 ppm + Sucrose 4%
T <sub>4</sub>	AgNPs coated	8-HQS 225 ppm + AgNPs 20 ppm + Sucrose 4%

### 2.10. Statistical Analysis

All the data were statistically analyzed using a completely randomized design (CRD). A one way analysis of variance (ANOVA) was performed and the means were compared in each treatment, using the Duncan's new multiple ranges test (DMRT) (at a significance level of 0.05).

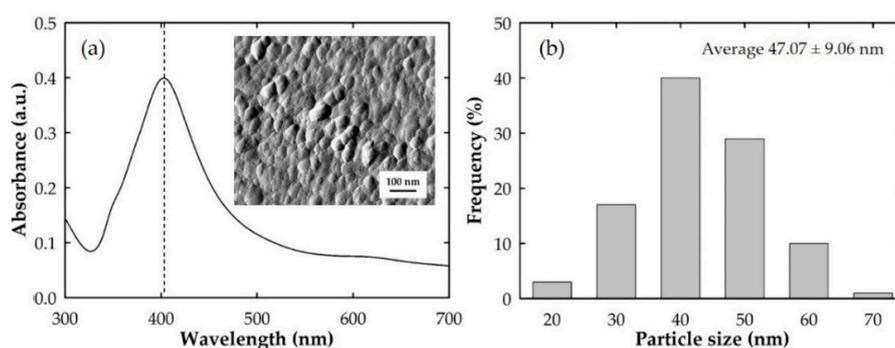
## 3. Results

### 3.1. Synthesis of AgNPs

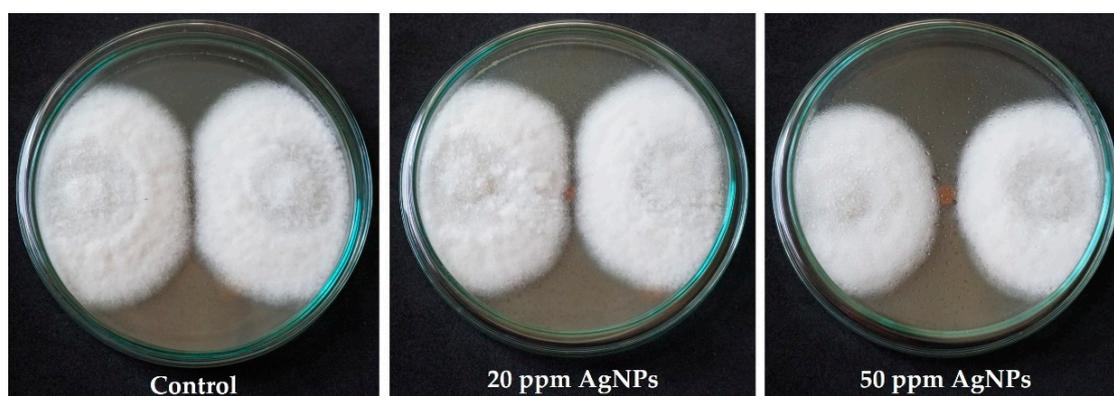
The synthesis of AgNPs was carried out using the chemical reduction of NaBH<sub>4</sub> and Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>. The formation of light yellow colored AgNPs in an aqueous solution was determined by UV-Vis spectroscopy, set to the absorbance mode of the surface plasmon resonance (SPR) peak. It was observed that the SPR peak in the UV-visible spectrum of the AgNPs was at a wavelength of 403 nm (Figure 1a). Generally, the absorption of AgNPs depends on the size of the particles [24]. Previous studies have reported that the absorption spectrum peaks at wavelengths between 400–430 nm and can be attributed to the size of AgNPs, which ranges between 20 and 60 nm [18,25,26]. The particle size of AgNPs was measured and further confirmed by an atomic force microscope (AFM). The size of synthesized AgNPs varied between 20 and 70 nm, with an average of  $47 \pm 9.06$  nm, and were topologically spherical in shape (Figure 1a,b).

### 3.2. Antifungal Activity of Coated Paper

In this study, the antifungal activity of paper against *C. Gloeosporioides*, coated with 20 and 50 ppm of AgNPs, was investigated by observing the growth of the fungal colony diameter. Results are shown in Figure 2 and Table 2. As seen in the figure, the growth of fungi in a paper coated with 50 ppm AgNPs was significantly inhibited ( $p < 0.05$ ), compared with a paper coated with 20 ppm, after an incubation period of 2 days. According to these results, a 50 ppm AgNP coating strongly reduced the fungal growth.



**Figure 1.** (a) UV-visible spectrum as measured by UV-Vis spectroscopy with an atomic force microscope (AFM) image ( $1 \times 1 \mu\text{m}^2$ ) of the synthesized AgNPs and (b) particle size distribution of the AgNPs.



**Figure 2.** Antifungal activity in a control plate (no AgNP coating) and test plate with different concentrations of AgNPs coated paper, for 7 days of incubation.

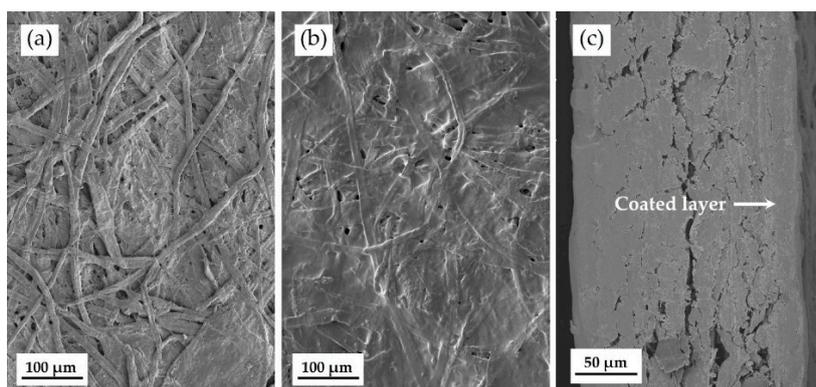
**Table 2.** Inhibition efficacy (% relative to control) of AgNPs coated paper (20 and 50 ppm) to *Colletotrichum Gloeosporioides* fungi.

Days	Inhibition (%Relative to Control)	
	AgNPs Coated Paper (ppm)	
	20	50
1	ND	ND
2	$12.00 \pm 2.70^a$	$36.00 \pm 2.18^b$
3	$3.92 \pm 1.79^a$	$18.95 \pm 2.73^b$
4	$4.88 \pm 2.44^a$	$11.22 \pm 2.99^b$
5	$4.26 \pm 1.73^a$	$10.08 \pm 2.21^b$
6	$2.72 \pm 1.42^a$	$6.80 \pm 3.27^b$
7	$0.00 \pm 0.00^a$	$3.33 \pm 1.83^b$

Data are presented as mean  $\pm$  standard deviation, followed by the same letters in the row, indicating that the numbers are not significantly different ( $p > 0.05$ , based on Duncan's new multiple ranges test (DMRT)), ND–Not determined.

### 3.3. Morphology of Uncoated and Coated Paper

FE-SEM micrographs of the surface of an uncoated paper indicated that the surface was rough and had a porous fibrous structure, as shown in Figure 3a. After coating the paper with a solution of AgNPs, at about  $4.0 \text{ g/m}^2$  of coating weight, the paper showed a relatively smoother surface compared to the uncoated paper (Figure 3b). This smoothness is probably a result of the coating solution filling the pores on the entire surface of the paper, making it homogeneous (Figure 3c) [27,28].



**Figure 3.** Field emission scanning electron microscope (FE-SEM) micrographs of (a) uncoated paper, (b) paper coated with AgNPs, and (c) cross-section of the surface coated with a layer of AgNPs.

### 3.4. Basis Weight and Thickness

Table 3 shows the basis weight, thickness, coated weight, and coating thickness of an uncoated and coated paper. The uncoated paper had a basis weight of  $134 \text{ g/m}^2$  and a thickness of  $174 \mu\text{m}$ , which were used as a baseline value. When the paper was coated with a coating bar (No. 3), the basis weight and thickness was  $137 \text{ g/m}^2$  and  $180 \mu\text{m}$ , respectively. This indicated that the weight of the coated paper increased by  $4 \text{ g/m}^2$ .

**Table 3.** The basis weight, thickness, coated weight, and coating thickness of the uncoated and coated paper.

Properties	Paper	
	Uncoated	Coated
Basis weight ( $\text{g/m}^2$ )	$133.88 \pm 1.25^a$	$137.61 \pm 1.34^b$
Thickness ( $\mu\text{m}$ )	$174.28 \pm 0.95^a$	$180.00 \pm 0.72^b$
Coating weight ( $\text{g/m}^2$ )	ND	$3.73 \pm 0.32$
Coating thickness ( $\mu\text{m}$ )	ND	$5.72 \pm 1.15$

Data are presented as mean  $\pm$  standard deviation, followed by the same letters in the row, indicating that the numbers are not significantly different ( $p > 0.05$ , based on the DMRT); ND—Not determined.

### 3.5. Tensile and Burst Strength

The tensile and burst strengths of uncoated and coated paper are shown in Table 4. The tensile index of the uncoated paper along the machine direction (MD) and cross machine direction (CD) was  $56.33 \pm 2.33$  and  $23.74 \pm 1.02 \text{ N}\cdot\text{m/g}$ , respectively. For the coated paper, the tensile index in MD and CD was  $56.02 \pm 3.56$  and  $25.94 \pm 0.98 \text{ N}\cdot\text{m/g}$ , respectively. The tensile index significantly increased with increasing coating weight in CD ( $p < 0.05$ ), while the tensile index did not change in MD. The bursting index of the uncoated paper was  $2.79 \pm 0.13 \text{ kPa}\cdot\text{m}^2/\text{g}$ , which significantly increased to  $3.08 \pm 0.08 \text{ kPa}\cdot\text{m}^2/\text{g}$  ( $p < 0.05$ ) when the paper was coated. This was due to the solution creating an excellent film on surface of the paper, which increased the bursting strength [29].

### 3.6. Water Absorptiveness

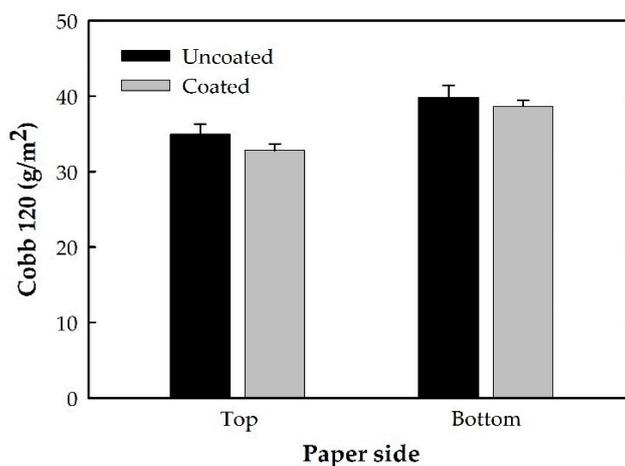
The water resistance of an uncoated and coated paper was determined using the Cobb test, and are shown in Figure 4. The resistance of the top side of a coated paper was significantly different ( $p < 0.05$ ) compared to an uncoated paper, and the value decreased from  $34.97 \pm 1.35 \text{ g/m}^2$  for the uncoated paper to  $32.81 \pm 0.86 \text{ g/m}^2$  for the coated paper. The water absorptiveness of the bottom side of an uncoated and coated paper was not significantly different ( $p > 0.05$ ), with the value decreasing from  $39.82 \pm 1.58 \text{ g/m}^2$  for the uncoated paper to  $38.65 \pm 0.78 \text{ g/m}^2$  for the coated paper. The results

indicate that the starch can reduce the water absorptiveness and can effectively improve the moisture barrier properties of hydrophilic films [30,31].

**Table 4.** The bursting and tensile index of the uncoated and coated paper.

Properties	Paper	
	Uncoated	Coated
Tensile index (Nm/g)	–	–
MD	56.33 ± 2.33 <sup>a</sup>	56.02 ± 3.56 <sup>a</sup>
CD	23.74 ± 1.02 <sup>a</sup>	25.94 ± 0.98 <sup>b</sup>
Bursting index (kPa·m <sup>2</sup> /g)	2.79 ± 0.13 <sup>a</sup>	3.08 ± 0.08 <sup>b</sup>

Data are presented as mean ± standard deviation followed by the same letters in the row, indicating that the numbers are not significantly different ( $p > 0.05$ , based on the DMRT).



**Figure 4.** Water absorptiveness values for the uncoated and coated paper as obtained from the Cobb's test.

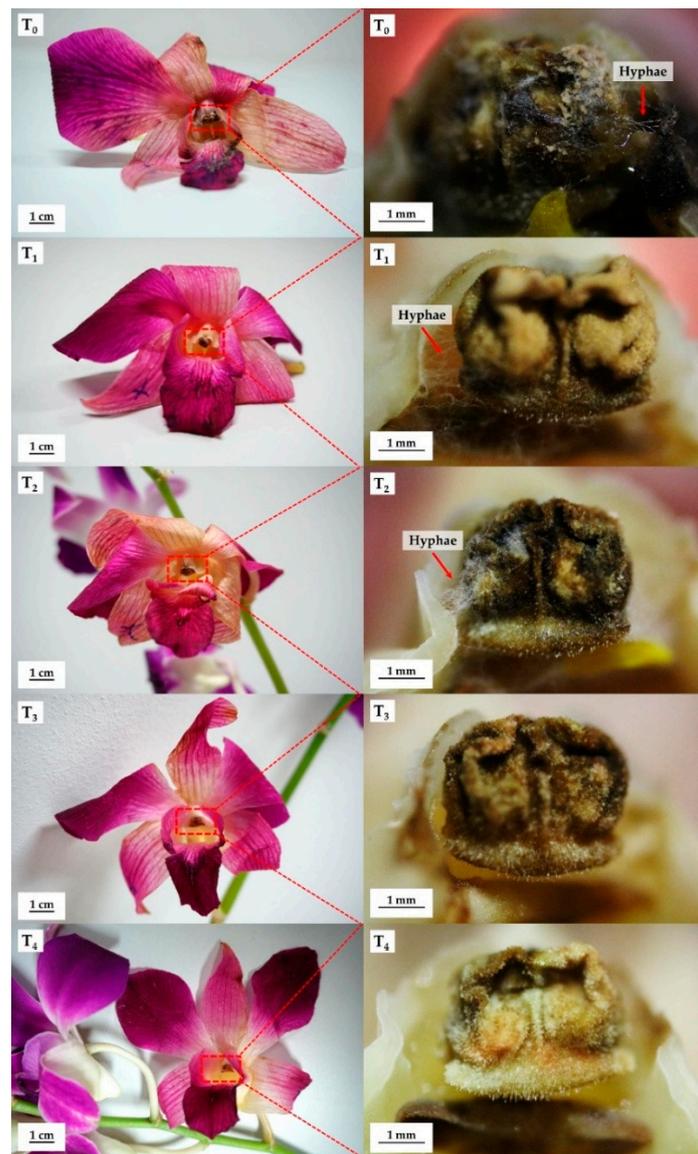
### 3.7. Effect of AgNP Coating on *C. Gloeosporioides*

Table 5 shows the percentage of infected orchid flowers during the packaging test, conducted for 7 days. The highest infection percentage was calculated at 40% for the T<sub>0</sub> treatment. While T<sub>3</sub> and T<sub>4</sub> treatments resulted in a lower infection percentage at 12.5% and significant difference ( $p < 0.05$ ) compared to T<sub>0</sub>, T<sub>1</sub>, and T<sub>2</sub> treatments. Therefore, a packaging coated with 50 ppm of AgNPs, combined with a pulsing solution (containing AgNO<sub>3</sub> and AgNPs), could inhibit the growth of *C. gloeosporioides* mycelium. Photographs of orchid flowers and their respective anthers are shown in Figure 5, used to further determine the hyphae of filamentous of *C. gloeosporioides* on the anther. Hyphae formations were found for T<sub>0</sub>, T<sub>1</sub>, and T<sub>2</sub> treatments. On the other hand, no *C. gloeosporioides* hyphae formation was observed on the anther for T<sub>3</sub> and T<sub>4</sub> treatments. This validated the speculation that a paper, coated with a concentration of 50 ppm of AgNPs, has good antifungal activity.

**Table 5.** Percentage of infected cut orchid flowers during the packaging test conducted for 7 days.

Treatments	Packaging	Pulsing Solution	Infection <sup>1</sup> (%)
T <sub>0</sub>	Uncoated	Distilled	40.00 ± 0.00 <sup>a</sup>
T <sub>1</sub>	Uncoated	8-HQS 225 ppm + AgNO <sub>3</sub> 30 ppm + Sucrose 4%	29.17 ± 1.44 <sup>a</sup>
T <sub>2</sub>	Uncoated	8-HQS 225 ppm + AgNPs 20 ppm + Sucrose 4%	31.67 ± 3.82 <sup>a</sup>
T <sub>3</sub>	AgNPs coated	8-HQS 225 ppm + AgNO <sub>3</sub> 30 ppm + Sucrose 4%	12.50 ± 0.00 <sup>b</sup>
T <sub>4</sub>	AgNPs coated	8-HQS 225 ppm + AgNPs 20 ppm + Sucrose 4%	12.50 ± 0.00 <sup>b</sup>

<sup>1</sup> Average ± standard deviation followed by the same letters in column indicating that the numbers are not significantly different ( $p > 0.05$ , based on DMRT).



**Figure 5.** Photographs of cut orchid flowers (left) and respective anthers (right), after being stored for 7 days during the packaging test. Hyphae formations can be observed for T<sub>0</sub>, T<sub>1</sub>, and T<sub>2</sub> treatments. On the other hand, no *C. gloeosporioides* hyphae formation is observed on the anther for T<sub>3</sub> and T<sub>4</sub> treatments.

#### 4. Conclusions

The AgNPs, synthesized in this study, which were spherical in shape and 47 nm in diameter, were prepared using chemical reduction and a stabilizing solution at 90 °C. Results from the UV-visible spectrography and AFM analysis confirmed the presence of AgNPs and their topology. The paper coated with 50 ppm of AgNPs had an excellent antifungal activity against *C. gloeosporioides*, under culture conditions. The AgNPs-starch coating increased the water resistance, tensile strength in CD, and bursting strength, of the paper. The paper coated with AgNPs, was successful in decreasing the infection caused by *C. gloeosporioides*, on cut orchid flowers. Therefore, AgNP coated paper has a great potential to be used widely in the packaging industry for producing boxes which can inhibit the *C. gloeosporioides* infection that plagues cut orchid flowers and other crops.

**Author Contributions:** Conceptualization, B.N. and B.P.; Methodology, B.N.; Software, B.N. and B.P.; Validation, B.N., S.P., S.K., and B.P.; Formal Analysis, B.N. and B.P.; Resources, B.N. and B.P.; Data Curation, S.P. and S.K.;

Writing-Original Draft Preparation, B.N. and B.P.; Writing-Review & Editing, B.N. and B.P.; Visualization, B.N. and B.P.; Supervision, B.P.; Project Administration, B.N.; Funding Acquisition, B.N. and B.P.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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