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Article

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

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Abstract: Metal nanoparticle has been reported to have a high antimicrobial activity against fungi, bacteria, and yeasts. In this study, silver nanoparticles (AgNPs) were synthesized using a chemical reduction method, at 90 °C, and used as an antifungal coating in paper packaging, to control the growth of *C. gloeosporioides* in cut orchid flowers during shipping. AgNPs were characterized by UV-Vis spectroscopy and atomic force microscope (AFM). The results indicated that the shape of AgNPs was spherical and homogenous with an average size of 47 nm. Twenty and 50 particles per million (ppm) concentration of AgNPs, mixed with starch, were prepared as the coating solution. The paper coated with 50 ppm AgNPs exhibited a significant antifungal activity against *C. gloeosporioides* compared to 20 ppm AgNPs coating. The AgNPs coated paper had a better water resistance and mechanical properties compared to paper without coating. We observed a significant reduction in the number of anthers, of orchid inflorescences, infected by *C. gloeosporioides*, when stored in the coated boxes. The current study demonstrates that paper boxes coated with AgNPs are a potential solution to control the infection of *C. gloeosporioides* in the storage of cut orchid flowers.

Keywords: Black anther disease; Orchid cut flower; Silver nanoparticles

1. Introduction

Orchid is one of the most important commercial ornamental plants in Thailand, especially the cut flowers and potted plants [1, 2]. Around 1300 species and 180-190 genera of orchid 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 trading in orchid around the world [2]. The total cut orchid flower export business is valued at around 59-70 million US$ (approximately 1,949-2,307 million Baht) and has been not stable during the last 5 years [2]. In addition, the exports of cut orchid flowers experienced a decrease by about 24.2 million tons valued at approximately 67.5 million US$ (approximately 2,228 million Baht), in 2017. Black anther, caused by the phytopathogen *Colletotrichum gloeosporioides*, is one of the major problems plaguing the postharvest quality of cut orchid flowers, especially during the rainy season [3, 4]. The symptoms of this disease include a black colored spot on the anther of an orchid cut flower [5], leading to a low quality and short vase life, and 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 orchid, during the postharvest shelf life, but their excessive use has caused the fungi to become resistant to fungicides. At the same time, there have been increasing concerns about the related consumer safety [5, 6]. Studies related to the development of an antimicrobial packaging paper, with properties that can prolong the shelf life during storage or transportation, while maintaining an acceptable quality, have 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 suitable for use with paper boxes in order to control the growth of *C. gloeosporioides*. However, the organic and natural biological antimicrobials were showed to be less stable at higher temperatures and high volatility, compared to inorganic ones [16, 17], which may result in only a limited application of the coated paper.

Therefore, the main objective of this study was to synthesize AgNPs by chemical reduction method and using 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. The antifungal activity of coated paper, to combat *C. gloeosporioides* by disc diffusion, were evaluated. The efficacy, of the antifungal packaging, in inhibiting the proliferation of black anther disease in stored cut orchid flowers, was also tested.

2. Materials and Methods

2.1. Materials

Uncoated paper (134 g/m^2), commonly used for orchid storage, 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 to prepare AgNPs, Silver nitrate ($AgNO_3$) and Sodium hydroxide (NaOH), were purchased from Merck Co. (Darmstadt, Germany). Trisodium citrate ($Na_3C_6H_5O_7$) and sodium borohydride ($NaBH_4$) 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 chemical synthesis method of Agnihotri et al. [18]. Briefly, a mixture aqueous solution containing 24 ml of sodium borohydride (2×10^{-3} mol dm^{-3}) and 24 ml of trisodium citrate (4.28×10^{-3} mol dm^{-3}) was heated to 60 ^{o}C for 30 min, in the dark. Two ml of silver nitrate (1×10^{-3} mol dm^{-3}) solution was then added to the mixture, and heated from 60 to 90 ^{o}C. The reaction was allowed to continue for an additional 20 min and then cooled to room temperature. UV–visible properties of the synthesized AgNPs solution were measured, using a spectrophotometer (UV-1800, Shimadzu Corp., Kyoto, Japan), at wavelengths ranging between 300–700 nm. The dimensions of AgNPs were examined by an atomic force microscope (MFP-3D (Bio), Asylum Research Corp., Santa Barbara, CA, USA).

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± 3 ^{o}C for 30 min. The obtained starch solution was cooled down to 65± 3 ^{o}C and the AgNP solution added in the desired quantity (0, 20, and 50 ppm) before use.

2.4. Preparations of antifungal coating papers

The blended solution was coated on multiple papers (180x180 mm) using the bar coating method. The coated papers were then dried in an oven at 105±2 ^{o}C for 15 min. All the coated papers were prepared at a constant coated weight of 4±0.5 g/m^2. The morphology of both the surface and cross-section of the paper samples were examined by 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 10 nm platinum coating.

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 measured 3, 5, and 7 days after incubation with the experiment being repeated 5 times. The percentage inhibition was calculated from the equation

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

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

2.6. Basis weight and thickness

Basis weight and thickness of the uncoated and coated papers was measured according to ISO 536: 1995 and ISO 534: 2005 standards, respectively. The weight of 10 individual papers was measured and the mean values calculated. The thickness of the papers was measured using a micrometer (Lorentzen & Wettres, Stockholm, Sweden). Each paper was randomly measured at different 5 positions and the mean thickness value, of a single paper, was calculated.

2.7. Tensile and bursting test

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

2.8. Water absorptiveness

The water absorptiveness of the uncoated and coated papers was determined using the Cobb method according to ISO 535: 1991 standard. The papers were cut into squares of size 14×14 cm and clamped inside the ring of a Cobb tester, having an area of 100 m^2. Hundred ml of distilled water was poured into the ring and allowed the water to be absorbed for 120 s. The excess distilled water was then poured out and the wet paper was placed between blotting papers in order to remove the excess surface water on the paper. The Cobb value was measured as the amount of distilled water in g/m^2 and the experiment was repeated 10 times.

2.9. Antifungal activity of the coated paper on the development of C. gloeosporioides in cut orchid flowers

The cut orchid flowers (*Dendrobium sonia*) were collected from Siamtaiyoo farm Co. Ltd. (Samut Sakhon, Thailand) to be used in the experiments. The healthy cut orchid flowers were selected with a long stem (45 cm), flower with approximately 7±1 blooms, and 5±1 buds per stem (export quality grade). The boxes used for shipping the flowers, of size of 52 cm high × 40 cm wide × 60 cm long, obtained from Siamtaiyoo farm Co. Ltd. (Samut Sakhon, Thailand), were used during the packaging test. The entire inside surface of the boxes was attached with the AgNPs coated sample papers, using a double sided tape. The 40 stems of freshly cut orchid flowers were prepared for the box packaging test, with and without AgNPs coating. From each box, one bloom of the flowers was selected and wounded on the anther by puncturing it using a sterilized pin. Twenty μL of spore suspension (106 spore/ml) was then dropped into the wound. The box samples were stored at a room temperature of 25±2 °C and 50 % RH for 7 days. Table 1 shows the treatments used during the packaging test with the experiment repeated 5 times. The percentage of infection was calculated from the equation

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

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_0	Uncoated	Distilled water
T_1	Uncoated	8 - HQS 225 ppm + $AgNO_3$ 30 ppm + Sucrose 4%
T_2	Uncoated	8 - HQS 225 ppm + AgNPs 20 ppm + Sucrose 4%
T_3	AgNPs coated	8 - HQS 225 ppm + $AgNO_3$ 30 ppm + Sucrose 4%
T_4	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 means were compared for significant difference in each treatment, using the Duncan's new multiple ranges test (DMRT) ($p<0.05$).

3. Results

3.1. Synthesis of AgNPs

The synthesis of AgNPs was carried out using the chemical reduction of sodium borohydride and trisodium citrate. The formation of light yellow colored AgNPs in an aqueous solution was evaluated by UV-Vis spectroscopy. It was observed that the UV-visible spectrum of AgNPs was obtained at a wavelength of 403 nm (Figure 1a). The free electrons, as a result of after AgNPs preparation, give rise to a surface plasmon resonance (SPR) absorption band because of the combined vibration of free electrons from AgNPs in resonance with a light wave [19]. Generally, the absorption of AgNPs depending on the particles size [20]. Previous reports revealed that the absorption peaks between wavelengths of 400-430 nm and can be attributed to AgNPs of size ranging between 20 and 60 nm [18, 21, 22]. The particle size of AgNPs was measured and further confirmed by atomic force microscope (AFM). The size of synthesized AgNPs varied from 20 to 70 nm, with an average value of 47±9.06 nm and the synthesized AgNPs were topologically spherical in shape (Figure 1b, 1c).

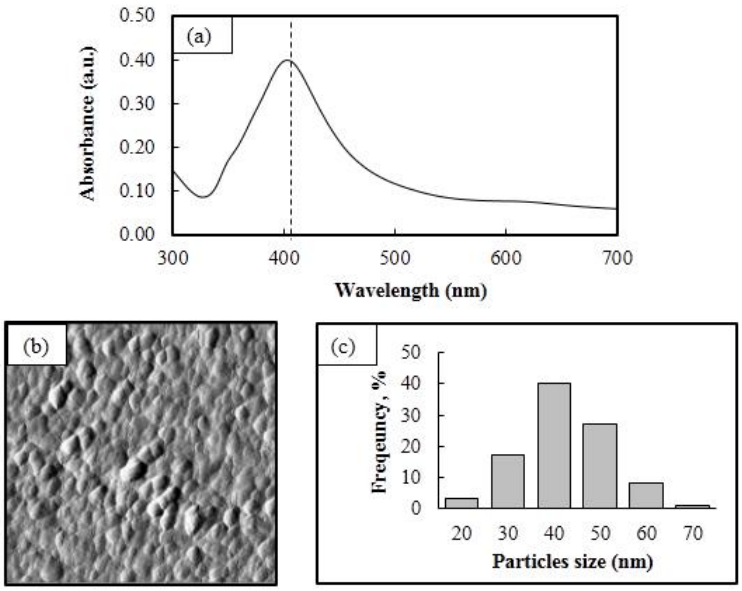

Figure 1. (a) UV–Visible spectrum as measured by UV-Vis spectroscopy, (b) AFM image (1×1μm) of the synthesized AgNPs, and (c) Particle size distribution of AgNPs.

3.2. Antifungal activity of paper coated by disc diffusion

In this study, the antifungal activity of paper coated with 20 and 50 ppm of AgNPs, was investigated, against *C. gloeosporioides*, using the growth of colony diameter method and results are shown in Figure 2 and Table 2. As seen in the figure, the growth of the fungi in a paper coated with 50 ppm AgNPs was significantly inhibited ($p<0.05$) compared with paper coated with 20 ppm, after incubation for 2 days. According to these results, a 50 ppm coating of AgNPs resulted in maximum inhibition of fungi on the coated paper.

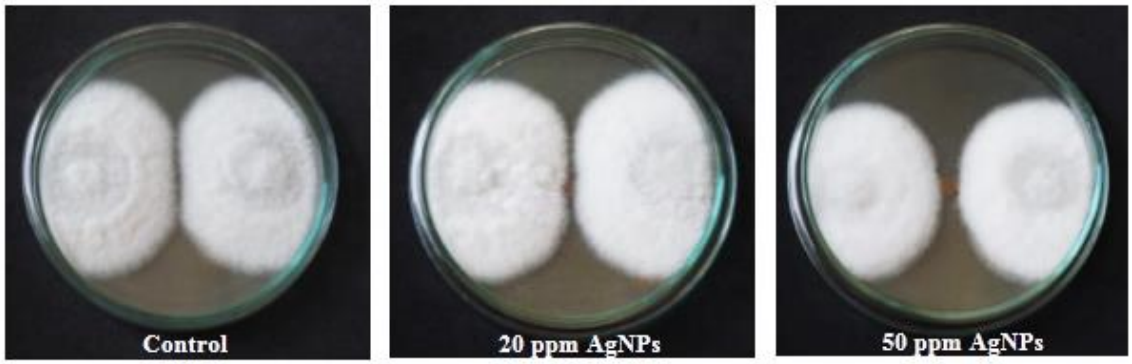

Figure 2. Antifungal activity in a control plate (no AgNP coating) and test plate with different concentrations of AgNPs coated paper, for 7 days and after 2 days of incubation.

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 containing AgNPs at about 4.0 g/m^2 the 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) [23, 24].

Table 2 Inhibition efficacy (% relative to control) of AgNPs coated paper (20 and 50 ppm) on *C. gloeosporioides.*

Days	Inhibition (%relative to control)[1]	
	AgNPs coated paper (ppm)	
	20	50
1	ND	ND
2	12.00±2.70^a	36.00±2.18^b
3	3.92±1.79^a	18.95±2.73^b
4	4.88±2.44^a	11.22±2.99^b
5	4.26±1.73^a	10.08±2.21^b
6	2.72±1.42^a	6.80±3.27^b
7	0.00±0.00^a	3.33±1.83^b

Remark: 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 DMRT), ND – Not determined.

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 g/m^2 and 174 μm thickness, which

were used as a baseline value. When the paper was coated with coating bar (No.3), the basis weight and thickness of was 137 g/m^2 and 180 μm, respectively. This indicated that the weight of coated paper increased by 4 g/m^2.

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

Properties	Paper	
	Uncoated	**Coated**
Basis weight (g/m^2)	133.88±1.25^a	137.61±1.34^b
Thickness (μm)	174.28±0.95^a	180.00±0.72^b
Coating weight (g/m^2)	ND	3.73±0.32
Coating thickness (μm)	ND	5.72±1.15

Remark: 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), ND –Not determined.

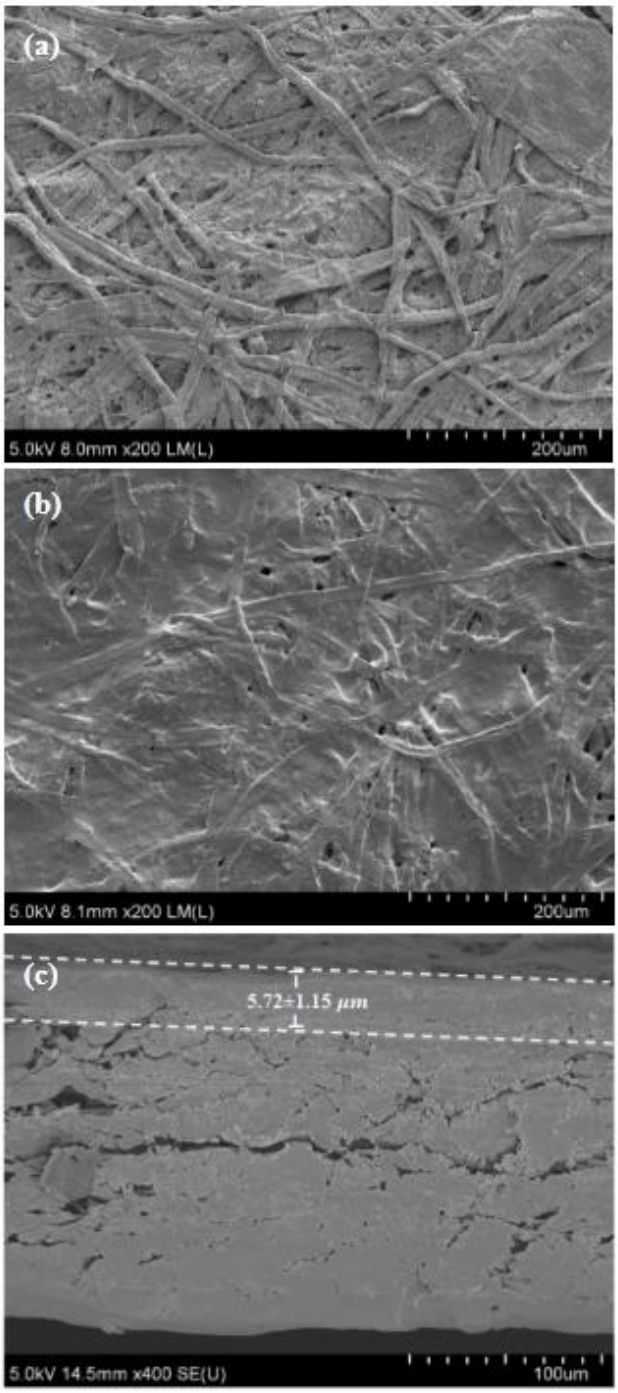

Figure 3. FE- SEM micrographs of (a) uncoated paper, (b) paper coated with AgNPs, and (c) cross-section of the surface coated with a layer of AgNPs.

3.5. 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±2.33 Nm/g and 23.74±1.02 Nm/g, respectively. For the coated paper, the tensile index in MD and CD were 56.02±3.56 Nm/g and 25.94±0.98 Nm/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 uncoated paper was 2.79±0.13 kPa m^2/g, which significantly increased to

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3.08±0.08 kPa m²/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 [25].

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[a]	56.02±3.56[a]
-CD	23.74±1.02[a]	25.94±0.98[b]
Bursting index (kPa m²/g)	2.79±0.13[a]	3.08±0.08[b]

Remark: 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).

3.6. Water absorptiveness

The results of water resistance test, of an uncoated and coated paper, using the Cobb test, are shown in Figure 4. The top side of the coated paper indicated to a significant difference ($p<0.05$) when compared with the uncoated paper, and the values decreased from 34.97±1.35 g/m² for the uncoated paper to 32.81±0.86 g/m² for coated paper. For the bottom side, the uncoated and coated paper, the water absorptiveness was not significantly different ($p>0.05$), with the values decreasing from 39.82±1.58 g/m² for the uncoated paper to 38.65±0.78 g/m² for the coated paper. The results indicate that the hydrophobic starch can reduce water absorptiveness, and can effectively improve the hydrophobicity and moisture barrier properties of hydrophilic films [26, 27].

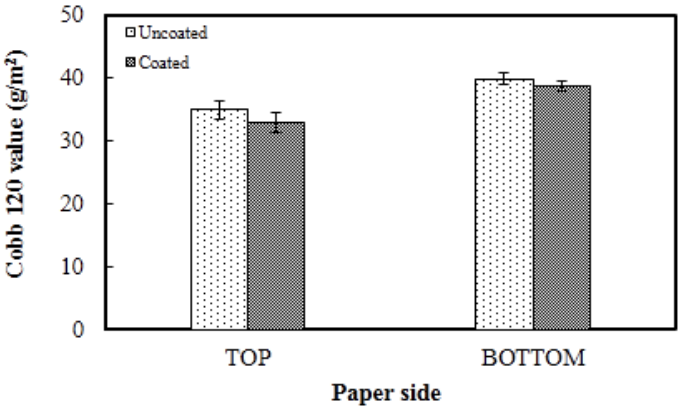

Figure 4. Water absorptiveness values for an uncoated and coated paper,as obtained from the Cobb's test.

3.7. Effect of AgNPs coating on C. gloeosporioides

Table 5 shows the percentage of infected orchid flowers during the packaging test conducted for 7 days. The highest level of infection percentage was calculated at 40% for the T_0 (control) treatment. While T_3 and T_4 treatments were found to have a lower infection percentage at 12.5% and significant difference ($p<0.05$) compared to T_0, T_1, and T_2 treatments. Therefore, packaging that was coated with 50 ppm of AgNPs combined with a pulsing solution (containing $AgNO_3$ and AgNPs) could inhibit the growth of *C. gloeosporioides* mycelium. Photographs of orchid flowers and their respective anthers are shown in Figure 5, to further determine the hyphae of filamentous of *C. gloeosporioides* on the anther. Hyphae formations were found for treatments T_0, T_1, and T_2. On the other hand, no *C.*

gloeosporioides hyphae formation was observed on anther for treatments T_3 and T_4. This validated the above speculation that the paper coated with AgNPs, at a concentration of 50 ppm, has a good antifungal activity with.

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

Treatments	Packaging	Pulsing solution	Infection[1] (%)
T_0 (control)	Uncoated	Distilled	40.00±0.00[a]
T_1	Uncoated	$AgNO_3$	29.17±1.44[a]
T_2	Uncoated	AgNPs	31.67±3.82[a]
T_3	AgNPs coated	$AgNO_3$	12.50±0.00[b]
T_4	AgNPs coated	AgNPs	12.50±0.00[b]

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

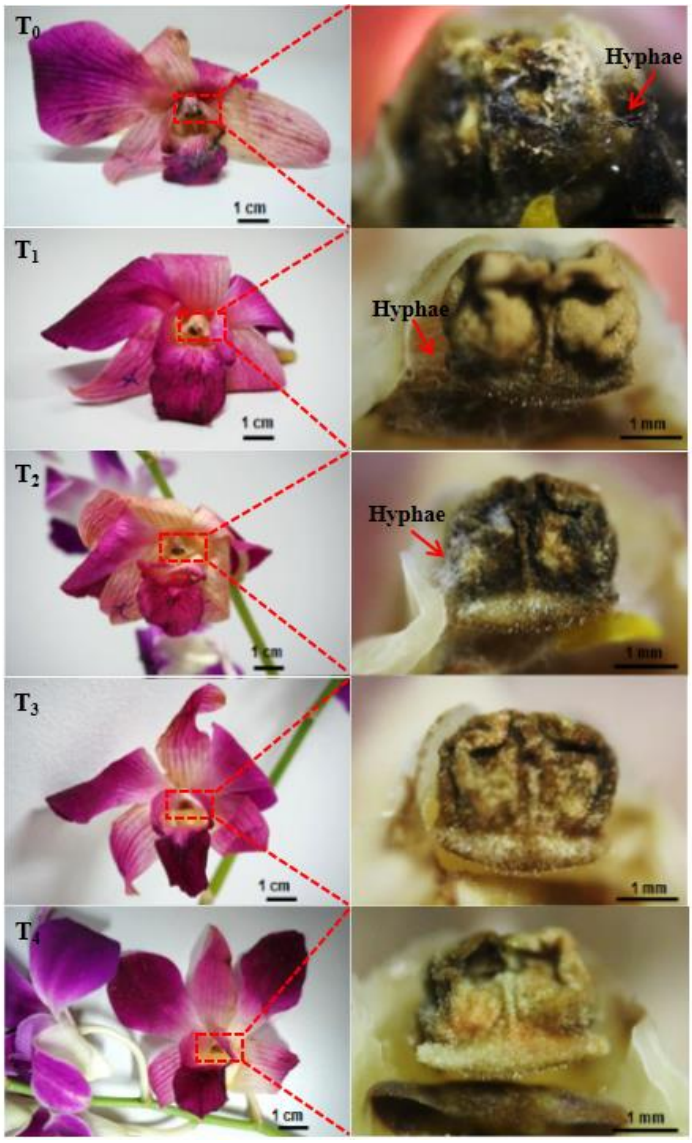

Figure 5. Photographs of cut orchid flowers (left) and anther (right) after stored for 7 days during the packaging test. Hyphae formations can be observed for treatments T_0, T_1, and T_2. On the other hand, no *C. gloeosporioides* hyphae formation was observed on anther for T_3 and T_4 treatments.

4. Conclusion

Synthesized AgNPs, spherical in shape and of size 47 nm, were prepared using chemical reduction and a stabilizing solution at 90 °C. Results from UV- Visible spectrography and AFM analysis confirmed the presence of AgNPs and their topology. Paper coated with 50 ppm of AgNPs had an excellent antifungal activity against *C. gloeosporioides,* under culture conditions. The AgNPs-strach coating increased the water resistance, tensile strength in CD, and bursting strength of the paper. After the packaging test, the resultant 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 applied widely in the production of paper products suitable for preventing *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 that they do not have any conflicts of interests.

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