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Inhibitory effects of Ag^+ and ZnO^+ nanoparticles on a causative agent (*Neoscytalidium dimidiatum*) of dragon fruit stem-canker

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ABSTRACT

Aims: This study aimed to isolate the fungal strains causing brown spot disease on dragon fruit and identify them using molecular biology techniques. The study also investigated the inhibitory effects of silver (Ag) and ZnO nanoparticles on the isolated fungal strains.
Methodology and results: Six fungal strains (TL1, TL2, TL3, TL4, TL5, TL6) causing brown spot disease (stem-canker) were isolated. TL1 and TL2 isolates were used for testing the antifungal features of nanoparticles. Nanoparticles were directly added to the PDA medium to make a solution with concentrations of 50, 75 and 100 ppm. The antifungal feature of nanoparticles was screened by inoculating with the fungal samples for 72 h. The inhibitory capacity of ZnO and Ag nanoparticles against fungal strains was then investigated. TL1 and TL2 samples were identified as *Neocytalidium dimidiatum* using the internal transcribed spacer (ITS) region. The study also revealed that silver nanoparticles were more effective than zinc oxide nanoparticles in inhibiting the growth of fungal strains that cause brown spot disease on dragon fruit. Specifically, ZnO nanoparticles had the highest inhibitory effect on TL2, 61.27% at 100 ppm and Ag nanoparticles gave the highest inhibitory effect on TL2, 85.83% at 100 ppm.
Conclusion, significance and impact of study: The research findings suggest that the use of Ag and ZnO nanoparticles may be an effective way to control brown dragon fruit spot disease. It may help in improving dragon fruit yield and aesthetic quality. As a result, it may help in reducing economic loss for farmers. However, further research is required.

Keywords: Brown spot disease, dragon fruit, *Neoscytalidium dimidiatum*, silver nanoparticles, zinc oxide nanoparticles

INTRODUCTION

Dragon fruit or pitahaya (*Hylocereus undatus*) belongs to the Cactaceae family. This tropical fruit is widely cultivated in Asia, especially in Vietnam, Thailand, Indonesia, the Philippines, China and Taiwan. Dragon fruits are rich in vitamins, minerals and antioxidant compounds (Lim *et al*., 2007; Jaafar *et al*., 2009). In Vietnam, dragon fruit is cultivated in Binh Thuan, Long An and Tien Giang provinces. Moreover, this kind of fruit is one of the key products for exportation in Vietnam due to its high nutrient and economic value. Approximately 80-85% of the annual production of dragon fruits in Vietnam was exported (Hoat *et al*., 2018). However, dragon fruit has faced various diseases and insect pests, including brown spot disease, anthracnose, bacterial soft rot diseases, oriental fruit fly (*Bactrocera dorsalis* H.) and guava fruit fly (*Bactrocera correcta* B.) (Hoat *et al*., 2018). Consequently, the consumption rate, especially in exportation, will be decreased due to the market loss in production yield, quality and price.

Brown spot disease is caused by the pathogenic fungi *N. dimidiatum.* It is considered that this opportunistic pathogen infects the host via the wound on the stem (Mohd *et al*., 2013). Subsequently, the appressoria is formed on the surface of the host before directly penetrating an epidermal cell (Fullerton *et al*., 2018). Previous works have described the symptoms of dragon fruit infected by *N. dimidiatum,* including slower development of fruit in the early stage and inducing circular sunken orange/red-brown canker and fruit internal brown rot (Mohd *et al*., 2013; Yi *et al*., 2015). The infection of *N. dimidiatum* has become an acute issue in many countries, such as the US, Israel, China, Indonesia, Malaysia and Thailand (Chuang *et al*., 2012; Xu *et al*., 2018). There are no specific fungicides, high-efficiency fungicides currently on the markets, or biological products as treatments for brown rot disease. As a result, the farmers were forced to abandon infectious dragon fruit, resulting in economic and labor-intensive losses (Hoat *et al*., 2018; Ratanaprom *et al*., 2021).

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Nowadays, nanotechnology is one of the most important tools for modern agricultural science, bringing many positive effects, contributing to clean, cost-effective agriculture and increasing productivity as well as quality (Kah and Hofmann, 2014). Nanoparticles with sizes ranging from 1 to 100 nm can be used for crop protection via two different mechanisms: (1) a protectant where the nanoparticles only protect plants, or (2) a carrier where the nanoparticles contain potential pesticides or some other active compounds (Elmer *et al*., 2018; Worrall *et al*., 2018). They can be applied directly to the plants or onto parts such as seeds, foliar tissue or roots (Kah and Hofmann, 2014; Sekhon, 2014). Some metal nanoparticles such as copper, silver, zinc oxide, iron and titanium dioxide were tested for their antagonistic effects on some pathogenic bacteria, viruses and fungi (Gogos *et al*., 2012; Kim *et al*., 2018) and archived positive results to be certified as potential anti-microorganism agents (Dimkpa *et al*., 2013).

Silver nanoparticles have a role as protectants for plant disease management (Worrall *et al*., 2018). In 2009, Min and coworkers (2009) demonstrated that silver nanoparticles could damage the mycelium to inhibit phytopathogen fungi, such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Sclerotinia minor*. The previous study reported that nanoparticles silver has an antifungal effect against the fungi *Alternaria alternata*, *S. sclerotiorum*, *Macrophomina phaseolina*, *Botrytis cinerea* and *R. solani* via well diffusion assay (Krishnaraj *et al*., 2012). In addition, silica-silver nanoparticles were reported to inhibit the growth of *Pseudomonas syringae* and *Xanthomonas campestris* pv. *vesicatoria* up to 100% (Worrall *et al*., 2018). Under greenhouse conditions, Ag-dsDNA-GO composites exhibit high antibacterial properties, even with Ag concentrations as low as 13 ppm (Ocsoy *et al*., 2013; Strayer *et al*., 2016). Zinc oxide nanoparticles act as protectants. They were approved by the US Environment Protection Agency (US-EPA) for use as herbicides and industrial preservatives. Moreover, they were also approved for use in pesticides as stabilizers with a concentration not over 15% (w/w or w/v) of the formulation (Kalia *et al*., 2020). Additionally, products containing zinc salts were popularized for bacterial inhibitory activity against many phytopathogens (Kalia *et al*., 2020). Zinc oxide nanoparticles have investigated the characteristic antibacterial aspects under *in vitro* conditions. It has been reported to control bacteria and fungi such as *A. alternata*, *B. cinerea*, *S. sclerotiorum*, *Rhizopus stolonifera*, *R. solani*, *Mucor* spp., *Fusarium oxysporum* and *Penicillium* spp. (Mallaiah, 2015). The ZnO-nCuSi is a non-phytotoxic composite material that was able to control citrus canker disease under field conditions (Young *et al*., 2018). Moreover, the inhibition efficiency of zinc oxide nanoparticles was higher than other copper-derived nanomaterials (Young *et al*., 2018).

Previous research has indicated that nanomaterials are potential agents against phytopathogens. Brown spot disease on dragon fruit caused by *N. dimidiatum* has threatened the agriculture and economy of nations. There are no specific fungicides or bioproducts to treat brown spot disease. Therefore, this study aimed to isolate and identify the fungi causing stem canker on dragon fruit and assess the *in vitro* effect of Ag and ZnO nanoparticles as antifungal agents.

MATERIALS AND METHODS

Collection of samples

The fungi samples were collected from stem cankers at the dragon fruit gardens in Long An province, Vietnam. Nanoparticles were purchased from AMS Science and Technology Joint Stock Company (Hanoi).

Isolation of *Neoscytalidium* spp.

The stem cankers of Dragon fruit with typical symptoms of brown spot disease were collected, and their surfaces were sterilized with 70% alcohol before collecting the samples. The samples were then cut into small blocks (1 × 1 cm), soaked in 70% alcohol for about 30 sec, rinsed with distilled water and dried on paper. They were placed onto Petri dishes containing PDA agar medium (200 g potato, 20 g D-Glucose, 15 g Agar) and incubated at 27-30 °C until the mycelium appeared. The pathogenic fungi isolation was carried out until the pure cultures were obtained. The fungal isolates were identified based on the characteristics of mycelium and spore via an optical microscope, as described by Yi *et al*. (2015) and Crous *et al*. (2006).

DNA extraction and identification of *Neoscytalidium* spp.

The six fungal strains were selected from the previous test for identification by molecular techniques consisting of the following steps: DNA extraction, ITS region amplification by PCR, PCR product checking by using electrophoresis and sequencing ITS region.

The DNA extraction was performed following these steps: (1) The mycelium of the fungal strain was put into a tube containing 50 µL lysis buffer (LB) with iron balls; (2) The sample was ground for 10 sec (30 times/sec) and repeated 3-4 times; (3)Then, the samples were incubated with 850 µL of LB at room temperature; (4) After 30 min, the mixtures were centrifuged at 12.000 rpm for 10 min; (5) 450 µL of supernatants were transferred to new tubes; (6) Next, the samples were mixed well with 650 µL of absolute alcohol and incubated at room temperature for 15-20 min; (7) The mixture was centrifuged at 12.000 rpm for 10 min and obtained the pellets; (8) The pellets were washed with 400 µL of 70% alcohol, centrifuged for another 5 min (12.000 pm) and collected the precipitates; (9) The products were dried at 60 °C for 10 min under a vacuum evaporator, resuspended in 100 µL of 0.1× TE, and stored at -20 °C.

Furthermore, the DNA samples were examined for quality by electrophoresis technique. Then, fungal strains were identified via the amplification of ITS region by PCR, using the primers ITS1 (5'-TCC GTA GGT GAA GCG G-

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3'), ITS4 (5'-TCC TCC GCT TAGA TAT GC-3') (White *et al*., 1990). The PCR was carried out with the initial denaturation at 94 °C (60 sec), followed by 35 cycles of denaturation at 95 °C (45 sec), annealing at 54 °C (45 sec), extension at 72 °C (1 min 20 sec) and final extension at 72 °C (2 min). PCR products were subjected to electrophoresis on 2% agarose gel and sequencing. The two fungal isolates were identified via the nucleotide BLAST tool on NCBI.

Inhibitory effect of nanoparticles against fungi isolates.

The assay was conducted using a modified method (Nguyen *et al*., 2017). Briefly, the ZnO and Ag nanoparticles were added to a heated PDA agar medium to make solutions of 0, 50, 75 and 100 ppm concentrations. The mixtures were then vortexed well and poured into sterilised Petri dishes. The mixtures were left until they were solidified before conducting further experiments. A block of isolated fungi (size of 1 × 1 cm) was placed on the center of the Petri dish, which was then incubated at 27-30 °C for three days. Subsequently, the diameter of fungal colonies was measured and recorded to evaluate the antifungal efficiency of nanoparticle solutions. The control samples were PDA medium with deionized water. The assay was in triplicate for each concentration.

The inhibitory percentage = (1 - d/D) × 100%

Where d is the diameter of fungal colonies under the treatment of nanoparticles, D is the diameter of fungal colonies under control.

Data analysis

The data were firstly processed with EXCEL 2016, then statistically analyzed by ANOVA via Minitab 16 software. Tukey's test was done to examine the significant difference among values with a significance level of $P<0.05$. All the values were illustrated as means ± SD.

RESULTS AND DISCUSSION

***Neoscytalidium* spp. isolation**

After 3 days of inoculation, six isolates were grown rapidly on a PDA agar medium, including TL1, TL2, TL3, TL4, TL5 and TL6. In morphological character, they had circular colonies, an entire margin, white to black pigmentation (after 5-6 days), and cottony aerial mycelia (Table 1). These macroscopic characteristics resemble previous reports (Chuang *et al.*, 2012; Mohd *et al*., 2013; Yi *et al*., 2015). The colony diameter was enlarged up to 7.50 ± 0.77 cm, which was similar to *N. dimidiatum* characteristics mentioned by Yi *et al*. (2015). Furthermore, the mycelia under microscopic view were branched, septate and had rod-shaped arthroconidia (Figure 1).

Identification of *Neoscytalidium* spp.

Among six isolated strains, TL1 and TL2 were selected for sequencing. PCR method was used to amplify the ITS region and used electrophoresis to check the results. PCR product length was apparent on agarose gel around 600 bp as the expected product length (Figure 2). Subsequently, DNA sequences were submitted to the nucleotide database of GenBank. TL1 and TL2 were identified as *N. dimidiatum* (MG865987), with 100% similarity and 100% query cover by using the nucleotide BLAST tool in the Nation Center for Biotechnology Information (NCBI).

The antifungal ability of ZnO nanoparticles

ZnO nanoparticles illustrated the inhibitory effect on *N. dimidiatum* after 72 h of incubation. Among the two isolates, TL2 resisted the most. At 50 ppm of

Table 1: Characteristics of fungal strains isolated from dragon fruit's stem cancers.

Isolate code	Colony pigmentation	Colony diameter (cm)	Mycelia	Condia
TL1	White to black	8	Branched, septate	Rod-shaped arthroconidia
TL2	White to black	8	Branched, septate	Rod-shaped arthroconidia
TL3	White to black	7.8	Branched, septate	Rod-shaped arthroconidia
TL4	White to black	7.8	Branched, septate	Rod-shaped arthroconidia
TL5	White to black	7.4	Branched, septate	Rod-shaped arthroconidia
TL6	White to black	6	Branched, septate	Rod-shaped arthroconidia

Table 2: Inhibition effect of nano ZnO on TL1 and TL2 strains.

Strains	Concentrations (ppm)		
	50	75	100
TL1	12.50 ± 12.50% [b]	8.33 ± 14.43% [b]	55.83 ± 17.74% [a]
TL2	41.67 ± 9.55% [a]	58.33 ± 9.55% [a]	61.67 ± 12.58% [a]

Note**:** Values represent means ± SD (standard deviation). Values with different superscript letters in a column were significantly different according to Tukey's test ($p<0.05$).

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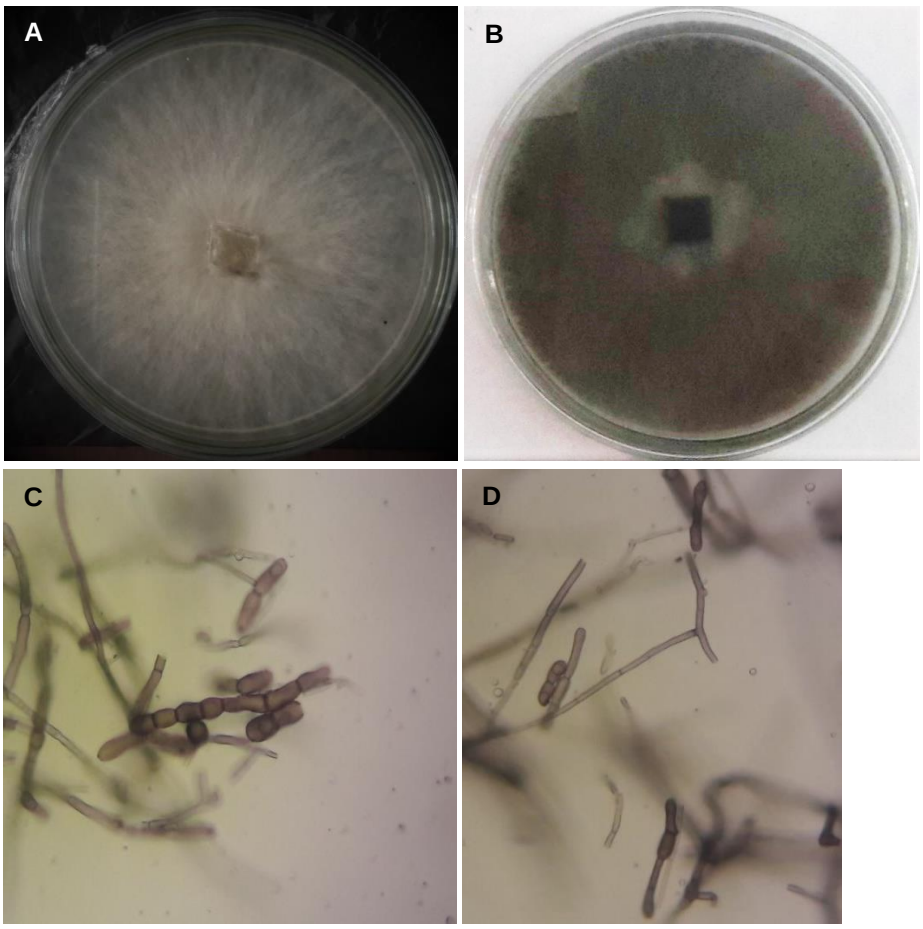

Figure 1: Macroscopic and microscopic characteristics of fungal isolates. (A) Colony and mycelium after 3 days incubation; (B) Colony and mycelium after 7 days incubation; (C) Microscopic view of fungal at 400× magnification; (D) Microscopic view of hyphae at 400× magnification.

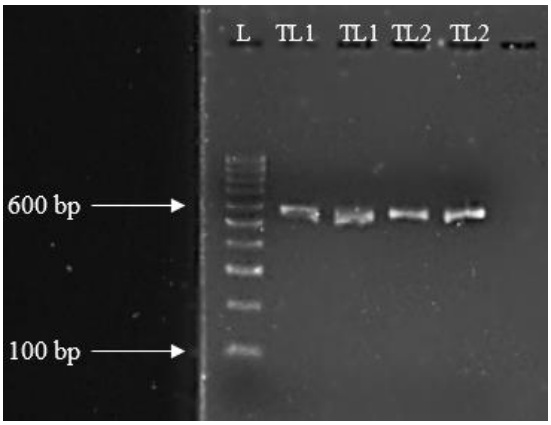

Figure 2: Agarose gel electrophoresis picture showing PCR products of ITS region. L, DNA Ladder (marker) 100 bp; TL1 and TL2, isolates (*Neoscytalidium* spp.).

nanoparticles, the inhibition affected on TL2 was three times higher than TL1, with 41.67 ± 9.55% and 12.50 ± 12.50%, respectively. Moreover, TL2 was restricted 50% of the growth by nanoparticles at 75 ppm, while TL1 was inhibited at the concentration of 100 ppm. Nevertheless, the antifungal efficiency against TL1 declined slightly at first, then rocketing nearly seven times from 8.33 ± 14.43% to 55.83 ± 17.74% at 100 ppm (Table 2). TL2 had a slight increase in inhibition effect with no statistical significance. Overall, ZnO nanoparticles' antifungal ability was more efficient on TL2 strain than TL1.

The antifungal ability of ZnO nanoparticles resulted from the formation of reactive oxygen species (ROS), which was caused by the redox activity of ZnO. The ROS induces oxidative stress that harms the cell's normal function (Yu *et al*., 2020). Remarkably, the high levels of oxidative stress possibly led to the inactivation of the metabolic pathway, gene mutation and cell death (Manke *et al.*, 2013; Yu *et al*., 2020). In 2011, Lipovsky *et al*. (2011) reported the antifungal activity of ZnO nanoparticles against *Candida albicans*, a species that is in the same division (Ascomycota) as *N. dimidiatum*. Notably, *C. albicans* was inhibited by ZnO nanoparticles with the minimum fungicidal concentration value (MFC) of

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Table 3: Inhibition effect of silver nanoparticles on TL1 and TL2 strains.

Strains	Concentrations (ppm)		
	50	75	100
TL1	30.42 ± 15.63% [b]	62.50 ± 0.00% [a]	76.67 ± 7.54% [a]
TL2	75.83 ± 3.82% [b]	85.83 ± 4.39% [a]	85.83 ± 3.15% [a]

Note**:** Values represent means ± SD (standard deviation). Values with different superscript letters in a column were significantly different according to Tukey's test (p<0.05).

0.1 mg/mL (100 ppm). Furthermore, De la Rosa-García *et al.* (2018) demonstrated that ZnO nanoparticles with the size of 50-70 nm (equivalent to the size of ZnO nanoparticles used in this study) were effectively resisted *Colletotrichum gloeosporioides* which causes anthracnose on avocado and papaya fruits. Additionally, *C*. *gloeosporioides* spore germination was inhibited by zinc oxide nanoparticles at a concentrations range of 0.156-0.625 mg/mL (156-625 ppm).

The antifungal ability of Ag nanoparticles

The effect of Ag nanoparticles on *N. dimidiatum* isolates was recorded after 72 h. The result of this test shared the same trend as ZnO nanoparticles. The inhibitory ability of Ag nanoparticle solution at concentrations of 50, 75 and 100 ppm ranged from 30.42 ± 15.63% to 76.67 ± 7.54% and ranged from 75.83 ± 3.82% to 85.83 ± 3.15% for TL1 and TL2, respectively.

The statistics from Table 3 revealed that TL2 was inhibited by more than 50%, even at the lowest treatment concentration of Ag nanoparticles, with 75.83 ± 3.82%. However, the results nearly remained constant despite the higher concentrations. As for TL1, the control capability has increased twice between 50 and 75 ppm and went up gently between 75 and 100 ppm. The test revealed the higher inhibitory activity of silver nanoparticles than zinc oxide nanoparticles. For instance, at 50 ppm, ZnO and Ag nanoparticles control the growth of TL1 12.50 ± 12.50% and 30.42 ± 15.63%, respectively. Similarly, two types of nanoparticles inhibit TL2 at 41.67 ± 9.55% and 75.83 ± 3.82%, respectively (Table 2).

The anti-pathogenic fungal ability of silver nanoparticles was based on the attack of the nanoparticles on the fungal cell membrane. According to Kim *et al*. (2008), silver nanoparticles performed on the membrane of fungi, disrupting the membrane potential and releasing intracellular components, which were essential for the survival of fungi.

Silver nanoparticles had a more noticeable antifungal effect than zinc oxide nanoparticles. It may be due to the performance of the particle size on pathogenic fungi attacks. Size is a vital factor involving the ability of nanoparticles to penetrate microorganism cells via the cell membrane. Additionally, silver nanoparticles and zinc oxide nanoparticles sizes were 10-20 nm and 50-70 nm, respectively. Nanoparticles are required to attach cell membranes successfully via receptors (active targeting) or permeation and retention at the injection site (passive targeting) (Wang *et al*., 2017). The study of Zakharova *et al*. (2015) reported that the smaller the particle size was, the more the toxicity grew because the increase in the number of surface atoms results in higher activity of nanoparticles due to unsaturated bonds.

CONCLUSION

Six fungal strains were successfully isolated from samples of stem brown spot disease of dragon fruit. Among them, the TL1 and TL2 were identified as *N. dimidiatum* based on morphological and molecular data. The silver nanoparticles and zinc oxide nanoparticles exhibited antifungal (*N. dimidiatum*) activity. However, the ability of silver nanoparticles to inhibit the TL2 strain (85.83%) was more effective than that of zinc oxide nanoparticles (61.27%). Both zinc oxide and silver nanoparticles could be used in developing commercial fungicides. However, further research is required.

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