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Study on the *In vitro* and *In vivo* Antifungal Activities of Nano-silver against *Mycoleptodiscus indicus* causing Leaf Blight on Lotus in Vietnam

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ABSTRACT

Background: Lotus, *Nelumbo nucifera* Gaertn is Vietnam's symbol and Buddhism's flower and plays an essential part in rural Vietnam's economy as all aspects of lotus could bring benefits to farmers. But, unfortunately, lotus yield in Vietnam is seriously affected by various plant diseases. Among them, leaf blight is currently emerging as one of the primary diseases devastating lotus crops in Vietnam, in which there are large necrotic parts on lotus leaves, flowers and seeds.

Methods: *Aloe barbadensis* extract was used for synthesizing silver nanoparticles. Leaf blight lotus leaves were collected to isolate pathogenic fungi-infection of isolated pathogenic fungi on the healthy lotus and then identify hidden mold by 28S rRNA sequencing. Determination of *in vitro* minimal inhibition concentration of nano-silver was conducted according to Azizi. The reduction of disease symptoms and biological characteristics of the treated lotus was observed.

Result: Morphological analysis and molecular identification of 28S rRNA sequencing showed that the pathogenic microorganism was *Mycoleptodiscus indicus* (*M. indicus*). Both *in vitro* antifungal activity and *in vivo* treatment of leaf blight lotus using a nano-silver solution showed that 30 ppm of nano-silver was the minimal inhibition concentration (MIC) for totally eradicating *M. indicus* growth. This was the first time *M. indicus* was reported to infect and cause leaf blight on a lotus. Previously, *M. indicus* was a well-known plant pathogen that could cross-kingdom infect humans and animals. Thus, the fact that lotus is widely cultured in Vietnamese rural could increase the chance for *M. indicus* to spread; hence, this raised the alarm about its potential harm to plants, humans and animals. And, significantly, it revealed nano-silver as a possible approach to prevent *M. indicus*.

Key words: Leaf blight, *Mycoleptodiscus indicus*, Nano-silver, *Nelumbo nucifera* Gaertn.

INTRODUCTION

Lotus, *Nelumbo nucifera* Gaertn, is the symbol of Buddhism. It is also a beautiful and helpful aquatic plant whose whole plant, including flowers, rhizomes, leaves, seeds and young branches. Lotus is used for a wide variety of purposes. Thus, lotus beautifies rural areas and plays an essential part in the rural economy for farmers in places with an abundance of damp soil, ponds and water submerged areas. It is a perennial aquatic macrophyte that has been cultivated for more than 2000 years in Asian countries and Australia, including India, Thailand, Australia, Vietnam, for its economic impacts, cultural values and medicinal uses. Vietnam and India elected lotus as the national flower (Zhu *et al*., 2019, Mekbib *et al*., 2020). Lotus was classified as a rhizome lotus with seed lotus and flower lotus based on their specific usages (Guo, 2009). Lotus was reported to perform a high degree of efficiency in photosynthesis and carbon conversion, which leads to a relatively high starch content in its seeds and rhizomes, thus contributing to local cuisine, culture and economy (Zhu *et al*., 2019).

Leaf blight can affect lotus and other crops, such as maize (Bruns, 2017; DeChant *et al*., 2017), rice (Chukwu *et al*., 2019), tomatoes (Adhikari *et al*., 2017; Esfahani, 2018), lily (Hong *et al*., 2016), kiwifruit (Adhikari *et al*., 2017), *Litchi chinensis* (Kumar *et al*., 2018), *Sansevieria trifasciata* (Kee *et al*., 2017) and *Ixora coccinea* (Banerjee *et al*., 2018). One of the suspected microorganisms causing leaf blight was *M. indicus,* which was reported to infect both plants, animals and humans (Garrison *et al*., 2008; Dewar and Sigler, 2010; Maboni *et al*., 2019). On the other hand, overuse of pesticides on leaves was found to leave an alarming amount of harmful residue on the produce, adversely affecting the consumers' health. Thus, there is a need to have a safe solution to prevent leaf blight while not harming the health of humans. To that extent, silver nanoparticles (AgNPs) have increased attention due to their attractive physicochemical properties, especially their anti-microorganism activity. During

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the time while carrying out previous research studies of lotus alkaloids, biodiversity in pink and white lotus in Hue City, Vietnam (Long *et al.*, 2019; Long *et al.*, 2020; Hong *et al.*, 2016; Trang *et al.*, 2019), researchers observed that leaf bright dramatically affected lotus fields here.

In this study, we synthesized silver nanoparticles using green *Aloe barbadensis* leaves to study the *in vitro* and *in vivo* antifungal activities of nano-silver against *Mycoleptodiscus indicus*, causing leaf blight on the lotus in Vietnam. We determined the inhibition effects of silver nanoparticles against *Mycoleptodiscus indicus* species under different concentrations *in vitro* and in the field trial. This study aims to find an optimal concentration of silver nanoparticles to inhibit the growth and development of *Mycoleptodiscus indicus* entirely and then use silver nanoparticles to prevent disease on lotus plants.

MATERIALS AND METHODS

Aloe barbadensis leaves are washed and finely cut into small pieces, then the chopped leaves are put in distilled water and boiled for 15 min, then allowed to cool naturally to room temperature. The solution was filtered and collected for green synthesis of silver nanoparticles and the leaf blight lotus (*Nelumbo nucifera*) was collected from high yield lotus fields on Hue City, Vietnam.

Green synthesis of silver nanoparticles using *Aloe barbadensis* extract

Ten grams of silver nitrate ($AgNO_3$) were dissolved in 500 mL of deionized water. Then, an aqueous solution of silver nitrate was mixed with 500 mL of plant extract from the *Aloe barbadensis*. This mixture was stirred for 15 min at room temperature in a dark condition. Finally, all of the solutions were heated in a microwave (2,4GHz, 800W). The reduction of Ag+ to Ag0 was confirmed by the color change of the solution from colorless to yellow, reddish-brown, or UV-Visible spectroscopy. Subsequently, the AgNPs solution was cooled down and diluted with 62,5 L ultrapure water to get an AgNPs solution of 100 ppm. All the AgNPs batches were prepared in duplicate.

Characterization of the silver nanoparticles

UV-Visible (UV-Vis) absorption measurements were performed using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) from 300 to 700 nm and operated at a resolution of 1 nm. Scanning electron microscopy (SEM) analysis was used to ascertain the morphology and size of the biosynthesized AgNPs.

Collecting leaf blight lotus leaves

Leaf blight lotus leaves were collected from a high yield lotus field. Leaves with large necrotic parts were collected. Diseased parts were dry brown, necrotic parts with yellow halos. They had unspecified shapes; some stood as single disease spots while the others might be joined together. Leaf blight lotuses were collected from high yield lotus fields on Hue City, Vietnam.

Young diseased leaves were picked to obtain strong fungal samples.

Isolation of pathogenic fungi

Small pieces (approximately 2×2 mm) of diseased leaf tissue from margins of individual lesions were surface disinfected in 1% sodium hypochlorite solution for 5 min, rinsed in sterile water, plated on water agar and incubated at 25°C. After 3 days, mycelium was isolated, transferred to potato dextrose agar (PDA) and then incubated at 25°C in a 12-h light/darkness regimen. Pathogenic fungi were isolated and kept in stock on slant PDA (Qiu, Yang *et al.*, 2015; Gurung, Dasila *et al.*, 2020). Fast-growing colonies on PDA were white to orange or pink with abundant acervuli but no perithecium. One-celled conidia were ovoid to oblong and 12 to 20×4 to 6 (15.9×5.0) µm.

Infection of isolated pathogenic fungi on healthy lotus

Isolated fungus was cultured in liquid culture. Wounds 1 cm × 1 cm were made on healthy leaves of a 7-week-old healthy lotus tree. Then these wounds were infected with pathogenic fungal liquid culture. Appearances of leaf blight on lotus were observed (Kim *et al.*, 2001; Choi *et al.*, 2017).

Identification of isolated mold by 28S rRNA sequencing

Pathogenic fungi were sent to Nam Khoa Company for molecular identification. After DNA extraction, 28S rRNA sequence was amplified by PCR, aligned and compared to similarities with specific sequences at the National Center for Biotechnology Information (NCBI) (Sandhu *et al.*, 1995).

Determination of *in vitro* minimal inhibition concentration of nano-silver

An antifungal assay was performed on PDA. After wet sterilization, the PDA was cooled down to 60°C and added to an appropriate amount of nano-silver solution to obtain serial dilutions of nano-silver on the PDA (Azizi, Pourseyedi *et al.*, 2016). Then pathogenic fungus was inoculated at 3 points on PDA plates followed by inverted incubation at 30°C-32°C. Inhibition of nano-silver on the development of pathogenic fungi was observed.

In vivo antifungal activities of nano-silver

Healthy lotus plants were artificially infected by the pathogenic fungi. When leaf blight appeared, infected lotus were directly sprayed with nano-silver solution at MIC found in previous experiments. The reduction of disease symptoms and biological characteristics of treated lotus was observed (Kim *et al.*, 2001).

Data analysis

All experiments were triplicated. Data were analyzed by SPSS 20.0.

RESULTS AND DISCUSSION

Observation of color change and UV-Vis spectroscopy

The successful synthesis of AgNPs using of *Aloe barbadensis* extracts were confirmed by color changes and

spectroscopic analysis. After stirring, the color of the mixture of $AgNO_3$ and plant extracts changed from pale yellow to reddish - brown in 15 min, revealing the conversion of ionic silver (Ag^+) to metallic silver (Ag) and then into colloidal particles (AgNPs). Ag nanoparticles were concentrated and purified by centrifugal ultrafiltration and then rinsed and dried. Formation of Ag nanoparticles was indicated by theappearance of signature brown colour of the solution (Fig 1). This was the first indication of the efficient synthesis of AgNPs. This observation is consistent with the previous reporting that silver ions are reduced in the presence of plant extracts due to the reducing properties of secondary metabolites (polyphenols, sterols, alkaloids, terpenoids, flavonoids, proteins, *etc.*).

The mechanism of Ag^+ reduction reaction to silver nanoparticles is as follow:

$$4Ag^+ + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ag^0 + C_6H_5O_7H_3 + 3Na^+ + H^+ + O_2\uparrow$$

In this reaction, the presence of *Aloe barbadensis* extract played a role by acting as a stabilizing agent while controlling the silver nanoparticles. Results of electron microscopy analysis showed that silver nanoparticles had quasi-spherical shapes in size range of 5 nm-40 nm (Fig 2).

The wavelength of plant extract nanoparticles was measured using UV-visible Spectroscopy to ascertain the formation of AgNPs. The presence of a strong absorbance peak at about 415 nm clearly indicated the formation of AgNPs due to the surface plasmon resonance (SPR) electrons phenomena present on the nanoparticle surface (Fig 3).

Fig 1: Aqueous synthesized silver nanoparticles.

Morphology of isolated pathogenic fungi

Leaf blight areas commonly appear at the edge of the leaf veins, or they might spread over the leaf surface (Fig 4). Leaf blight pathogenic microorganisms were isolated on the PDA. Pathogenic fungi formed fast-growing, thick and smooth white mycelia colonies on PDA plates with black spores at the center. Fungal colonies were yellow, as seen from the bottom view (Fig 5A, 5B). Microscopic morphology of isolated fungi showed that this fungus was cellular with branched hyphae. It produced circular spores attached to the end of hyphae (Fig 6A, 6B). The colony morphology and microscopic morphology of the isolated fungi were similar to those of *M. indicus*, which cause rare infections in humans and animals (Maboni, Krimer *et al.*, 2019). Morphology of the fungal colonies was also similar to *M. indicus* isolated from diseased *Ixora coccinea* (Banerjee, Mandal *et al.*, 2018).

Infection of isolated pathogenic fungi on healthy lotus

Healthy 7 -week-old lotus leaves were artificially wounded and infected with isolated fungi. After five days, the appearance of leaf blight was observed. The wound spread, turning reddish-brown and the leaf surface became necrotic (Fig 7). The appearance of leaf blight symptoms on the lotus reconfirmed that isolated fungus was the pathogenic cause of this disease on a lotus.

Identification of isolated mold by 28S rRNA sequencing

DNA extraction, PCR for 28S sequencing, analyzed by Basic Local Alignment Search Tool (BLAST) and compared to sequences of those of the NCBI. Those sequences noted were 100% similar to sequence JE05849, which included 614 nucleotides (score 1134) of *M. indicus*. This finding showed that the isolated pathogenic fungus was *M. indicus.* This was the first time *M. indicus* was reported to cause the leaf blight infection on the lotus (Fig 8).

In vitro* antifungal activity and MIC of nano-silver against *M. indicus

People usually use synthetic chemicals to treat diseases on plants and crops. However, this process has led to contamination by harmful residue chemicals of produce. On the other hand, nano-silver is being used to treat deadly

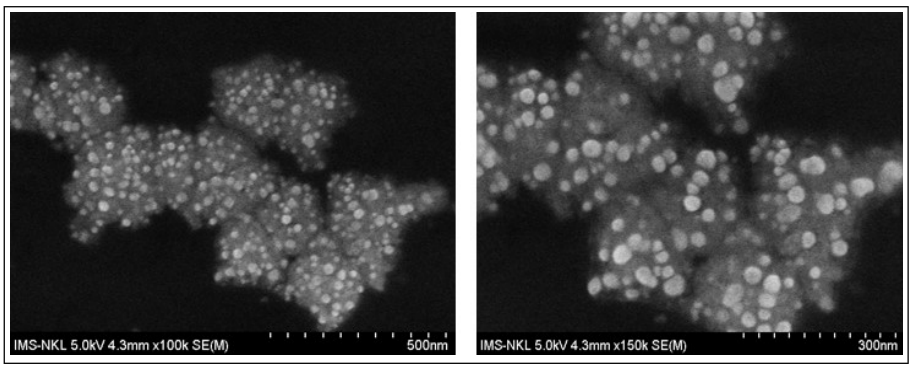

Fig 2: Scanning electron microscopic (SEM) images of silver nanoparticles synthesized by *Aloe barbadensis* leaf extract.

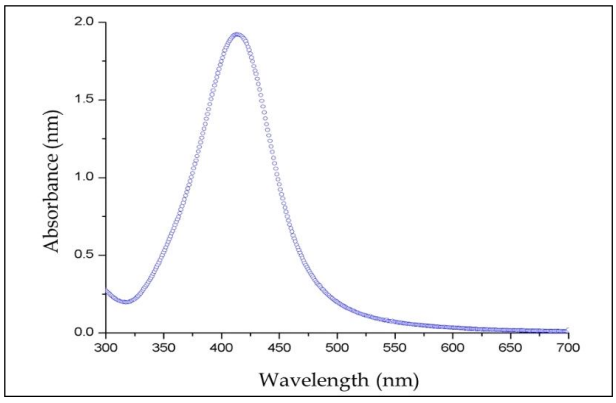

Fig 3: Absorption spectra of nanoparticles. Start wavelength: 300 nm, stop wavelength: 800 nm.

diseases on these plants in recent times, which has led to higher yields with less harm to human health. Thus, the choice to treat fungi infections on the edible lotus is a safer solution for leaf blight.

Serial dilutions prepared from nano-silver 40 ppm PDA plates against *M. indicus* were observed (Fig 9). It was found that increasing silver nano concentration led to more significant and more reliable inhibition of *M. indicus.* From a concentration of 1 ppm to 10 ppm, the development of *M. indicus* has slightly reduced this growth compared to the control plate. However, concentrations of nano-silver higher than 15 ppm significantly inhibited *M. indices'* growth. Impressively, *M. indicus* colonies were tiny spots at concentration 25 ppm and they disappeared at the concentration of 30 ppm. This finding showed that 30 ppm

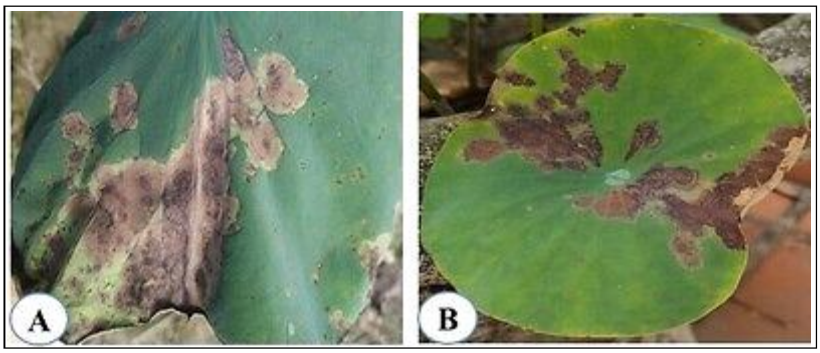

Fig 4: Leaf blight lotus with large necrotic areas in (A) mature leaves and (B) young leaves.

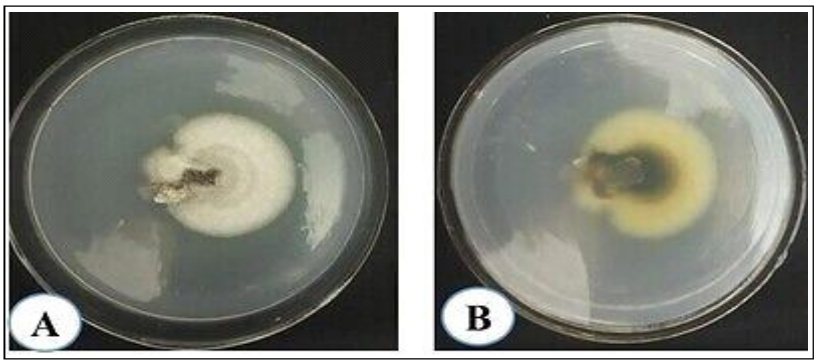

Fig 5: Pathogenic fungal colonies on PDA plates. A. Top view. B. Bottom view.

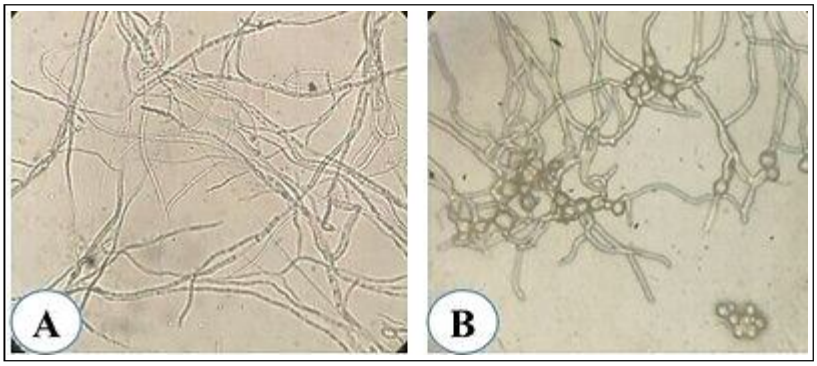

Fig 6: Microscopic morphology of isolated fungi. A. Mycelia (10×40). B. Spore (10×1000).

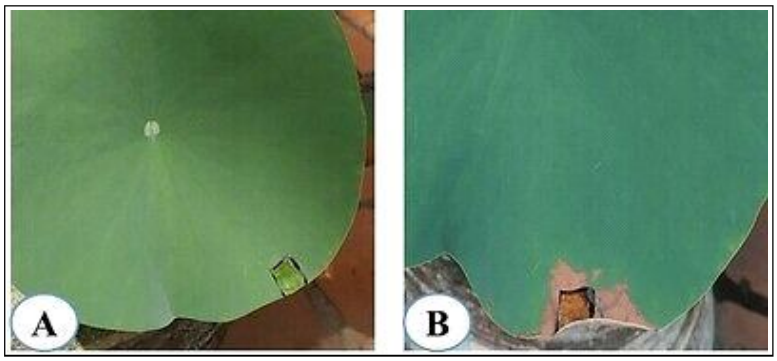

Fig 7: Infection of isolated pathogenic fungi on healthy lotus. A. Wounds on lotus leaves. B. leaf blight appeared after 5 days.

Score	Expect	Identities	Gaps	Strand
1134 bits(614)	0.0	614/614(100%)	0/614(0%)	Plus/Minus

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Query  1    GATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCGACGTTTGAAAGTCGGG  60
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Query  61   TCGTTGTCCGGCGGGCGCGTGGTCGCCTGGAAGCCGCATGTCTGCTGCGCTCCGGGCTGA  120
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Sbjct  554  TCGTTGTCCGGCGGGCGCGTGGTCGCCTGGAAGCCGCATGTCTGCTGCGCTCCGGGCTGA  495

Query  121  CACCAGCACCGCCGATGCATTTAAGGGCCGCCGCCGTATCTCAGGGGCGATCCCCAACAC  180
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Sbjct  494  CACCAGCACCGCCGATGCATTTAAGGGCCGCCGCCGTATCTCAGGGGCGATCCCCAACAC  435

Query  181  CAAGCTTGCGCTTGAGGGGCGTAACGACGCTCGAACAGGCATGCCTCGAGGAATACCAAG  240
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Sbjct  434  CAAGCTTGCGCTTGAGGGGCGTAACGACGCTCGAACAGGCATGCCTCGAGGAATACCAAG  375

Query  241  AGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAAATCTGCAATTCACACTAGTTA  300
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Query  301  TCGCATTTCGCTGCGTTCTTCATCGATGCCGGAGCCAAGAGATCCATTGTTAAAAGTTGT  360
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Query  601  CGGAAACCTTGTTA  614
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Sbjct  14   CGGAAACCTTGTTA  1
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Fig 8: Results from BLAST sequence analysis and sequence on NCBI.

was an *in vitro* MIC of nano-silver for its inhibition when applied to *M. indicus*.

In vivo antifungal activity of nano-silver

In vitro MIC of nano-silver - 30 ppm - was applied to treat leaf blight *in vivo* on artificially induced leaf blight of lotus leaves (Fig 10). Wounds with a size of 1 cm × 1 cm were made on lotus leaves, followed by spraying of *M. indicus* liquid culture directly on newly wounded areas. The first treatment was 8 hours after infection. Pathogenic symptoms appeared after 8 hours, leading to a spreading of white areas around the initial wound. Next, the 30 ppm of silver nano was sprayed on diseased leaves. Amazingly, the diseased parts did not expand for 24 hours and remained unchanged until five days later. However, after five days, the diseased areas developed necrosis and turned reddish-brown (Fig 10).

On the other hand, the second treatment was carried out 24 hours after developing an *M. indicus* infection. Leaf blight areas (white areas with purple halos) expanded widely on infected leaves after 24 hours of infection. Then this sample was sprayed with 30 ppm nano-silver. The same phenomenon in the previous sample was observed: the leaf blight area stopped expanding and only became necrotic after five days. These impressive inhibitions of leaf blight demonstrated that 30 ppm of nano-silver could inhibit the growth of the pathogenic fungi *M. indicus* on lotus leaves.

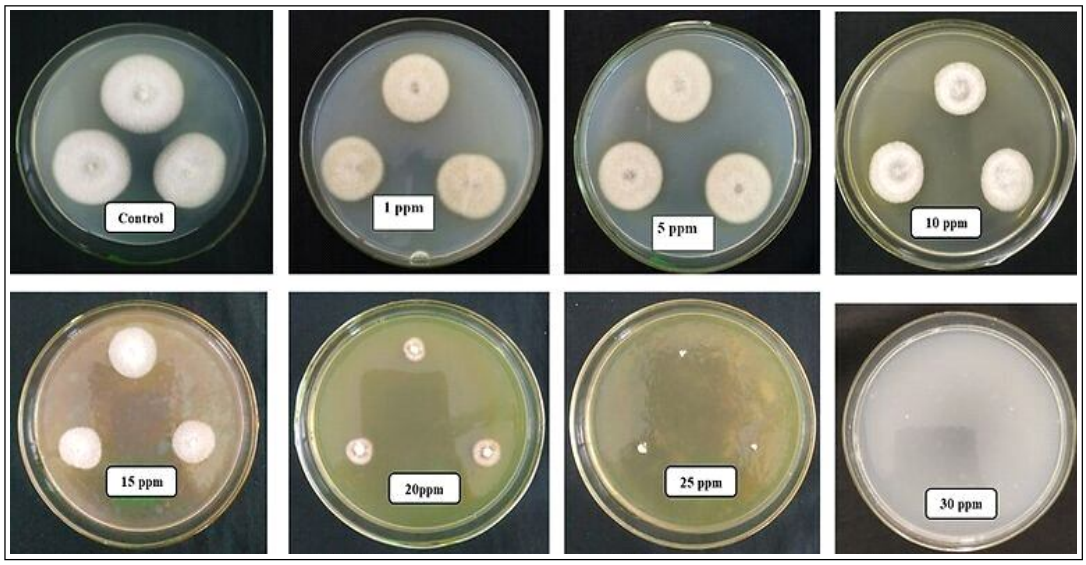

Fig 9: *In vitro* antifungal activity of nano-silver against *M. indicus*.

Mycoleptodiscus indicus is widely known to cause pathogens on many plant parts, causing leaf blight and twig dieback on Ixora coccinea (Ostazeski, 1967; Banerjee *et al*., 2018). On Zamia, *M. indicus* caused leaf necrosis with reddish-brown death spots on leaves (El-Gholl and Alfieri, 1991) similar to the necrotic parts on lotus leaves in this current study. Moreover, *M. indicus* is responsible for some diseases on a wide variety of living creatures such as an orchid tree in Malaysia, passion flowers in China, Grape, Ivy, *etc*.); animals (cats, dogs in the US, *etc*.) and humans (Maboni *et al*., 2019). In 2019, an 8-month-old immunocompetent cat in Georgia, USA, was diagnosed with an infection on the front knee with *M. indicus* (Maboni *et al*., 2019). It also caused several dermal excoriations in an 8-year-old immunodeficient dog (Metry *et al*., 2010).

Several cases of *M. indicus* infection in humans have been reported. In patients with compromised immune systems, the presence of *M. indicus* can lead to this opportunistic pathogenic microorganism causing an infection. For example, it was observed to cause a proliferation of skin nodules with a sporotrichosis lymphangitic distribution in a 51-year-old patient who had just undergone liver transplantation and incidentally suffered from a concurrent infection of HIV and HCV. More seriously, this pathogenic fungus invaded his veins and caused an angioinvasion when performed a histologic analysis (Garrison *et al*., 2008). In 2010, a 54 year old Canadian man was reported to have septic arthritis of his left knee caused by an infection with *M. indicus* after his three-week vacation to Costa Rica (Dewar and Sigler, 2010). In addition, a leg infection with *M. indicus* was found in a 72-year-old gardener who was also immunodeficient and demonstrated the presence of granulomatosis (Padhye *et al*., 1995). In 2012, a glioblastoma multiforme patient was also reported to be infected with necrotizing lesions of his leg caused by *M. indicus* (Koo *et al*., 2012). These cases were diagnosed with the awareness of the possibility of *M. indicus* infections in immunodeficient patients who proved to be compatible hosts of this fungus.

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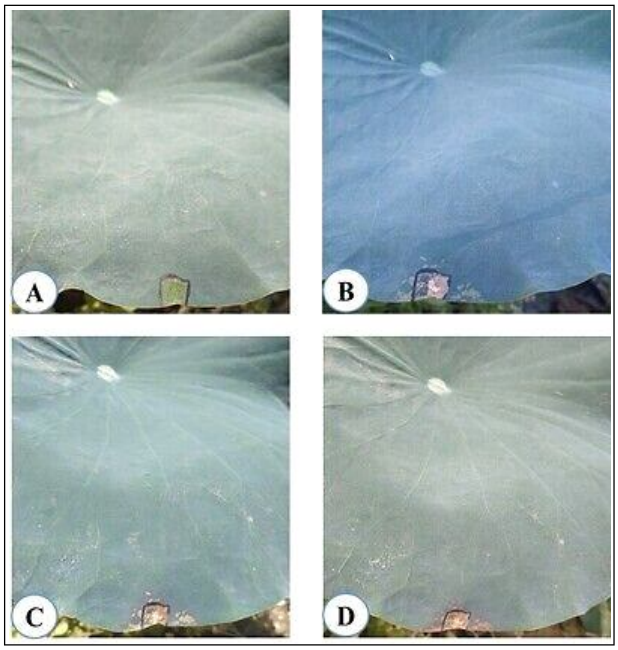

Fig 10: *In vivo* antifungal activity of nano-silver after 8 hours of *M. indicus* infection. A. Wound was made on healthy lotus leaves for the purpose of observing the development of *M. indicus* on them. B. Leaf blight appeared after 8 hours. C. Leaf blight area after 24 hours treated with 30 ppm nano-silver. D. Leaf blight area after 5 days treated with 30 ppm nano-silver.

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CONCLUSION

M. indicus was proven to be the pathogenic microorganism responsible for leaf blight on lotus crops. It is well known as a pathogenic fungus that causes diseases in plants, humans and animals. Fortunately, 30 ppm of nano-silver was found to inhibit its growth in *vitro* and *in vivo*. Therefore, it could be concluded that nano-silver might be a potential solution to treating *M. indicus*. However, before a nano-silver solution can be officially applied as a treatment for *M. indicus,* further studies on its activities and safety for humans, plants and animals should be carefully addressed.

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