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Evaluation of Silver Nanoparticles Toxicity against Toxic Black Mold *Stachybotrys chartarum*

Marwah MB*

Academic Campus for Girls, Jazan University, Jazan, Kingdom of Saudi Arabia

Abstract

Stachybotrys chartarum is very common in buildings and homes and will grow anywhere indoors where there is moisture. Therefore, in the current study it was isolated from walls with excessive moisture covered with susceptible paint. As an alternative to synthetic fungicides, the use of silver nanoparticles (AgNPs) as antifungal agents has become more widespread. Ag-NPs exhibited a potent antifungal activity against *S. chartarum*, similar to the antifungal activity of chemical fungicide Carbendazim. Synergistic action was reported when AgNPs was added to chemical fungicide. The inhibition zone in case of 5 ppm of Carbendazim was 12 mm but the addition of 25 ppm, 50 ppm, 75 ppm and 100 ppm of AgNPs increases the inhibition zone to 20 mm, 26 mm, 34 mm and 36 mm respectively. Nine bands of DNA with different molecular weights 8900 bp, 7700 bp, 4600 bp, 2200 bp, 1100 bp, 900 bp, 750 bp, 500 bp and 300 bp were detected in *S. chartarum* at 50 ppm of AgNPs while one band was detected in untreated fungus with molecular weight 9300 bp indicating that the AgNPs causes DNA fragmentation. SDS-PAGE gel electrophoresis was carried out to monitor the change in gene expression of *S. chartarum* exposed to AgNPs where the protein bands (15 bands) appeared in control and treated *S. chartarum* except band number 3 with molecular weight 15.0 KD was detected only in control and shifted to 16.0 KD in treated fungus. Finally, AgNPs application to building materials and walls could effectively protect indoor environments from mould development.

Keywords: Evaluation; Silver nanoparticles; Toxicity; *Stachybotrys chartarum*

Introduction

The presence of fungi as well as other microorganisms in indoor environments causes numerous serious diseases and acute or chronic toxicological syndromes. In order to inhibit or prevent the growth of fungi on building materials, the disruption of their vital processes or the reduction of reproduction as well as sporulation is required [1-3]. *Stachybotrys chartarum* (*S. chartarum*) is the most common fungus found in homes or buildings which have sustained flooding or water damage from broken pipes, roof, wall or floor leaks and condensation. *S. chartarum* is a common bioindicator of moisture problems. It is often referred to as "toxic black mould" and is linked with sick building syndrome (SBS) [4]. According to many authors, *S. chartarum* has been isolated from pipe insulation, gypsum wallboard, glass fiber wallpaper, and aluminum foil in homes, with the addition the authors stated that, soiled surfaces and surfaces covered with susceptible paper or paint may facilitate fungal growth in the absence of dampness [1,5]. Residents of buildings highly affected with *S. chartarum* manifested adverse health effects due to the numerous bioactive metabolites such as macrocyclic trichothecenes, related trichoverroids, phenylspirodrimanes (spirolactones and spirolactams) and cyclosporins (potent immunosuppressive agents) produced by this fungus [6-9]. Respiratory, circulatory, and nervous systems of human are affected due to prolonged exposure to these metabolites. Other negative impacts are skin irritation and non-specific hypersensitivity reactions and the cancer development [10].

Nanoparticles (NPs) are those particles which have a size less than 100 nm in diameter and show unique chemical, physical and biological properties which is very much different when in large size [11,12]. NPs are of interest because of their two main properties such as chemical reactivity and optical behavior. Several authors [13,14], reported that silver ion is considered an attractive material for its distinctive properties, such as good conductivity, chemical stability, catalytic activity, and antimicrobial activity.

Antifungal activity of silver nanoparticles (AgNPs) has been reported by some authors [15-17]. Compared with other metals, silver exhibits higher toxicity to microorganisms while it exhibits lower toxicity to mammalian cells [18]. AgNPs attach to cell membrane and penetrate in the fungi then produce a site with little molecular weight in center of fungi, and then Ag-Nps attach to respiratory sequence and finally cell division stops which lead to cell death, Ag-Nps release silver ion in fungal cell which increase antifungal function [19]. Sang et al. [20] and McDonnell and Russell [21] hypothesized that the silver ions mostly affect the function of the membrane bound enzymes, for example those in the respiratory chain. Feng et al. [22] reported that DNA loses its ability to duplicate when the fungal culture was treated with Ag^+, which may lead to a deactivated expression of ribosomal subunit proteins and to the synthesis of disabled enzymes and cellular proteins, important for the adenosine triphosphate production. The spread of *S. chartarum* in homes and buildings and lack of published studies on the fungus control was the main reason for the aim of this study, which included the determination of the influence of AgNPs on the mould growth with mechanisms of AgNPs action.

Material and Methods

Silver nanoparticles and fungicide

AgNPs were obtained from Sigma Aldrich. According to the product insert, the particles size was less than 100 nm and was spherical in shape. Different concentrations of AgNPs (25, 50, 75 and 100 ppm) were used to study the effect on fungal growth. The chemical fungicide Carbendazim (50% SC) was used at different concentrations alone and with AgNPs as control.

Isolation and identification of *Stachybotrys chartarum*

S. chartarum was isolated from walls with excessive moisture of building material in Jazan region. The fungal isolate was purified by sub-culturing on Czapek-Dox agar medium. The identification of the fungal culture was done on the basis of the microscopic and macroscopic characteristics. The fungus colony of isolate was observed using hand lens and the colony morphology was recorded with respect to color, shape, size and nature of colony. The microscopic characteristics were recorded using software for image analysis in Jazan University, KSA. The fungus morphology was recorded with respect to spore chain morphology, hyphae and mycelium structure. The fungus was identified according to Ellis [23] and Domsch et al. [24].

Antifungal activity of silver nanoparticles and fungicide

The antifungal activity was studied using the well diffusion method as described by Swarup et al. [25]. The antifungal activity was assessed by seeding 0.1 mL of *S. chartarum* spores suspension on Czapek-Dox agar medium plates of 2.5 mm thickness. wells were made on agar surface with a sterile 5 mm cork borer. Different concentrations of AgNPs and chemical fungicide (20 µl) were added in the well. One cavity was considered as testing for control by filling up with the double distilled water. The plates were incubated at 25°C for 7days and the zone of inhibition was recorded.

Estimate of increase in fold area

The increase in fold area was measured by calculating the mean surface area of the inhibition zone of chemical fungicide and chemical fungicide + AgNPs according to Birla et al. [26]. The fold increase area of *S. chartarum* for chemical fungicide and for chemical fungicide + AgNPs was calculated by the equation $(B^2 - A^2)/A^2$, where A and B were zones of inhibition for antifungal chemical fungicide and chemical fungicide + AgNPs, respectively.

Protein gel-electrophoresis

S. chartarum was cultivated in broth medium supplemented with AgNPs at 25°C for 8 days. After incubation period of *S. chartarum,* their mycelia were separated from broth medium and 4 grams were ground in a volume of 0.1 mL sodium dodecyl sulfate. At 95°C the homogenates were heated for 5 min, then centrifuged at 12, 000 rpm. The obtained supernatants were analysed by Polyacrylamide Gel Electrophoresis. The extract was separated by electrophoresis on 1 mm thick 12.5% acrylamide slab gels and then stained with Coomassie blue [27] at Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt.

Effect of silver nanoparticles on fragmentation of DNA

S. chartarum was cultivated in treated and untreated broth medium with AgNPs at 25°C for 8 days to obtain fungus mycelia. Two hundred µl cell lysis buffer (50 mM TrisHCl, pH 8.0, 10 Mm ethylene diamine tetra acetic acid, 0.1 M sodium chloride, and 0.5% sodium dodecyl sulphate) were used to treat *S. chartarum* mycelia for 1 h at 37°C. proteinase K (0.2 mg/mL) was added to the lysate mycelia and incubated for 2 hrs at 40°C. At 10, 000 rpm and for 10 min the sample was centrifuged. The aqueous portion containing DNA was used to evaluate fragmentation process on 1% agarose gel containing 1 µg/mL Ethidium bromide [28] at RCMB.

Results and Discussion

Current building materials, when exposed to high relative humidity in areas of poor or inappropriate lightning, heating or ventilation, are frequently found to be excellent substrates for mould formation.

Therefore, in the current study *S. chartarum* was isolated from walls with excessive moisture covered with susceptible paint (Figure 1). As the wet conditions increased the growth of *S. chartarum* increased. This knowledge about *S. chartarum* was confirmed with many authors, where Nelson [29] reported that S. *chartarum* has been isolated from gypsum wallboard, pipe insulation, glass fiber wallpaper, and aluminum foil. Other studies [5,30] reported that soiled surfaces and surfaces covered with susceptible paint or paper may facilitate *S. chartarum* growth in the absence of dampness.

Antifungal activity of Ag-NPs toward *S. chartarum* was investigated in the current study, where the AgNPs exhibited a potent antifungal activity against it, the similar antifungal activity of chemical fungicide Carbendazim (50% SC), which was used as a positive control (Figure 2) was also reported. These results indicated that AgNPs have remarkable potential as an antifungal agent in controlling *S. chartarum* growth on walls. The high susceptibility of *S. chartarum* to AgNPs is surprising, considering fungi in general, have known to be resistant to various disinfectants. Nanoparticles have a high surface to volume ratio which changes their properties when compared to non-nanoscale forms of the same material. Moreover, nanoparticles are able to penetrate biological membranes and cell walls more effectively, leading to cell death [31]. The aggregation of nanoparticles drastically decreases their accessibility, resulting in insufficient functionality against microorganisms [32]. For this reason, homogeneous distribution of nanoparticles over building materials is required to guarantee better contact and reaction with microorganisms. In general, the inhibition zone of *S. chartarum* increased with the increase of the AgNPs concentration (Table 1) except 25 ppm of AgNPs exhibited no antifungal activity. These results are in agreement with those obtained from Min et al. [33]. Recently the antifungal activity
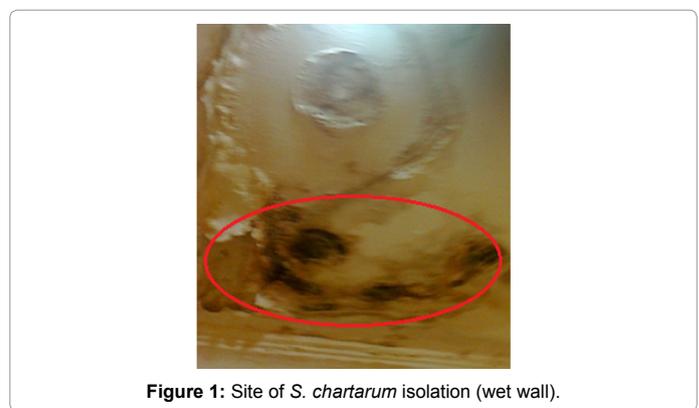

Figure 1: Site of *S. chartarum* isolation (wet wall).

Treatment	Concentration ppm	Inhibition zone (mm)
Control	0	00 ± 0.20
AgNPs	25	00 ± 0.10
	50	21 ± 0.40
	75	26 ± 0.50
	100	29 ± 0.11
Carbendazim	5	12 ± 0.08
	10	25 ± 0.02
	15	31 ± 0.12
	20	34 ± 0.07
Carbendazim (5ppm)+AgNPs	25	20 ± 0.11
	50	26 ± 0.05
	75	34 ± 0.40
	100	36 ± 0.20

Table 1: Inhibition zone of AgNPs and chemical fungicide Carbendazim and their combination against *S. chartarum*.

of AgNPs was tested against indoor mould *Chaetomium globosum* and *S. chartarum* grown on the surface of gypsum drywall [3]. It was found that the presence of AgNPs in concentrations of 30-200 mg/L significantly decreased the growth of the fungi. This may be due to AgNPs inhibit the mould sporulation process according to Pinto et al. [34].

The combined effects of AgNPs and antibiotics or commercial fungicides have been reported before [35], therefore in the current study synergistic action was reported when AgNPs was added to chemical fungicide. The inhibition zone in case 5 ppm of Carbendazim was 12 mm but the addition of 25 ppm, 50 ppm, 75 ppm and 100 ppm
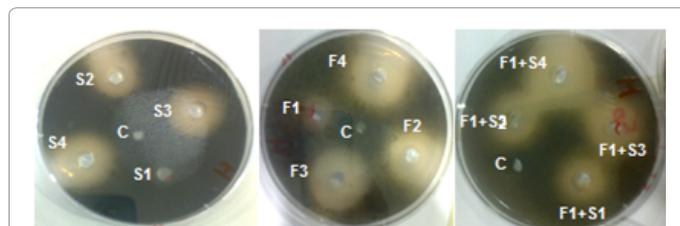

Figure 2: Photographs of inhibition zone of AgNPs against *S. chartarum* (C=control, S1=25 ppm AgNPs, S2=50 ppm AgNPs, S3=75 ppm AgNPs, S4=100 ppm AgNPs, F1=5 ppm Carbendazim, F2=10 ppm Carbendazim, F3=15 ppm Carbendazim, F4=20 ppm Carbendazim.
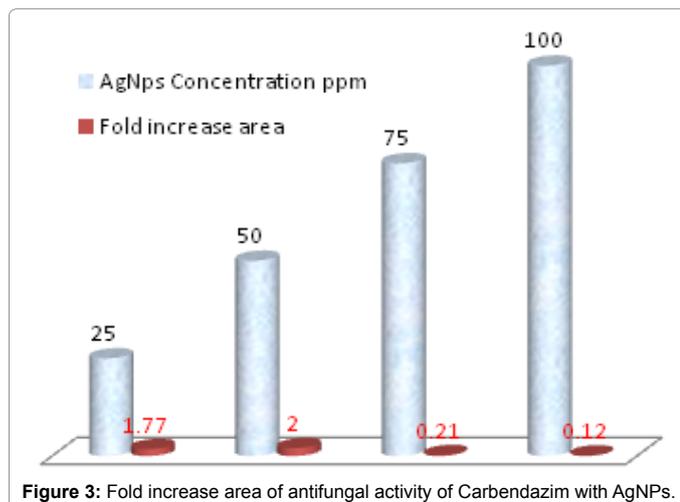

Figure 3: Fold increase area of antifungal activity of Carbendazim with AgNPs.
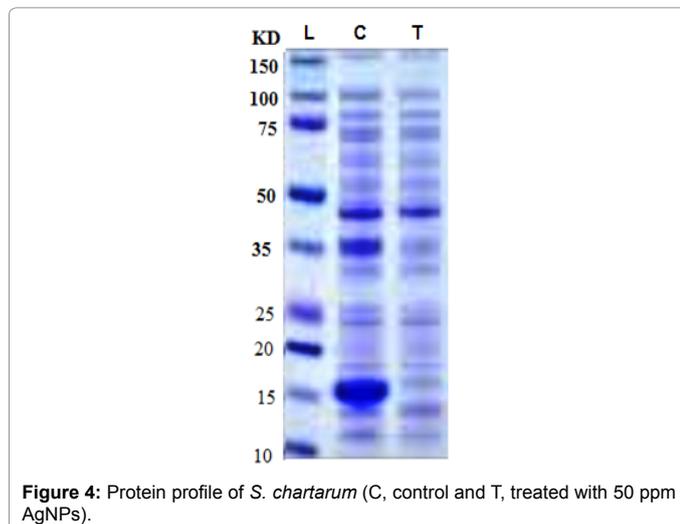

Figure 4: Protein profile of *S. chartarum* (C, control and T, treated with 50 ppm AgNPs).

Band No.	Molecular weight of protein bands (kd) of *S. chartarum*	
	Control	Treated with 50 ppm AgNPs
1	11.0	11.0
2	12.8	12.8
3	15.0	Shifted to 16.0
4	18.0	18.0
5	22.0	22.0
6	25.0	25.0
7	27.0	27.0
8	37.5	37.5
9	42.6	42.6
10	52.5	52.5
11	60.0	60.0
12	70.0	70.0
13	82.5	82.5
14	100	100
15	150	150

Table 2: Protein bands (kd) detected in *S. chartarum* (Control and treated with 50 ppm AgNPs).
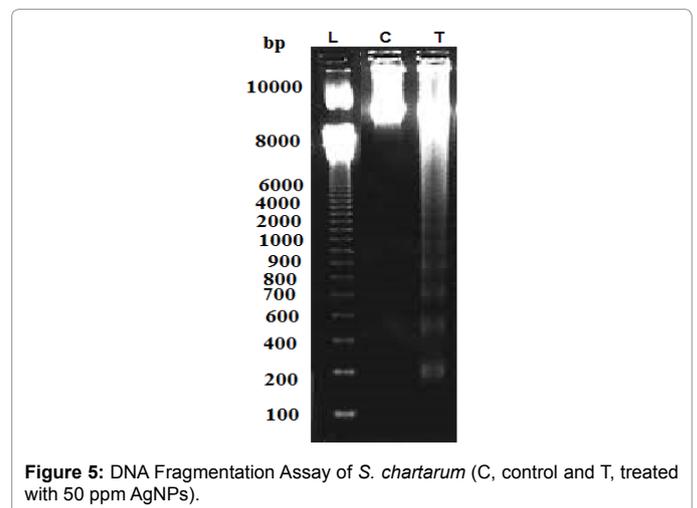

Figure 5: DNA Fragmentation Assay of *S. chartarum* (C, control and T, treated with 50 ppm AgNPs).

of AgNPs increase the inhibition zone to 20 mm, 26 mm, 34 mm and 36 mm respectively. These results are in agreement with the results obtained by Roy and Das [36] who reported that antifungal activities of the synthetic fungicides have been found to increase in the presence of AgNPs. Synergistic antifungal activity of Carbendazim with the different concentrations of AgNPs has been assayed on the basis of increase of fold area (Figure 3). It is clear that the increase of fold area was directly proportional with AgNPs concentration up to 50 ppm, then decreased. This indicated that the increase in AgNPs concentration didn't support the antifungal activity of Carbendazim. These findings corroborate with the report of Roy and Das [36].

To provide information on the mode of action of AgNPs, its ability to effect on proteins and DNA fragmentation were investigated in *S. chartarum*. Effect of AgNPs on proteins was detected with using Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (Figure 4). Although the growth of *S. chartarum* was inhibited by AgNPs but it was observed that the protein bands (15 bands) appeared in control and treated *S. chartarum* except band number 3 with molecular weight 15.0 KD was detected only in control and band 16.0 KD detected in treated fungus (Table 2). According to some researchers [15,37,38], AgNPs attach to the Sulphur containing proteins of the cell membrane, thereby causing membrane damage and depleting the levels of intracellular ATP of the microorganism. The cytotoxic effects of silver are the result of

Treatment with AgNPs	Molecular Weight of Fragmented Bands	
Ladder	10 K bp	
Control	9300 bp	
50 ppm	Band 1	8900 bp
	Band 2	7700 bp
	Band 3	4600 bp
	Band 4	2200 bp
	Band 5	1100 bp
	Band 6	900bp
	Band 7	750 bp
	Band 8	500 bp
	Band 9	300 bp

Table 3: DNA fragmented bands of *S. chartarum* (C, control and T, treated with 50 ppm AgNPs).

active physicochemical interaction of silver atoms with the functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA. In the present study, the genotoxicity exhibited by AgNPs was demonstrated by DNA fragmentation post treatment with 50 ppm of AgNPs (Figure 5). Nine bands with different molecular weights 8900 bp, 7700 bp, 4600 bp, 2200 bp, 1100 bp, 900 bp, 750 bp, 500 bp and 300 bp were detected at 50 ppm of AgNPs (Table 3) while one band was detected in untreated fungus with molecular weight 9300 bp. Previous study showed the same effect of AgNPs on DNA in the current study but in another fungus *Alternaria solani* [39].

It is evident that AgNPs directly interacts with macromolecular structures of living cells and therefore exerts an active influence on their physiology. Previous study by Cooke et al. [40] stated that AgNPS cause damage to the nuclear DNA by altering the chemical structure of the nucleotide bases and the deoxyribosyl backbone. This study was confirmed with the study of Damm et al. [41] who reported that silver can interact with the DNA of microorganisms, preventing cell division. Reports on the mechanism of inhibitory action of silver ions on microorganisms have shown that upon treatment with Ag$^+$, DNA loses its ability to replicate [22], resulting in inactivated expression of ribosomal subunit proteins, as well as certain other cellular proteins and enzymes essential to ATP production [42].

Conclusion

It could be concluded that, AgNPs can be used effectively in the control of *S. chartarum*. Current results support the hypothesis that AgNPS are suitable for formulating new types of fungicidal materials. Their application to building materials could effectively protect indoor environments from mould development.

Acknowledgment

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