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Biosynthesis of nanoparticles by fungus *Monacrosporium thaumasium* and its action on egg masses of the snail *Biomphalaria glabrata*

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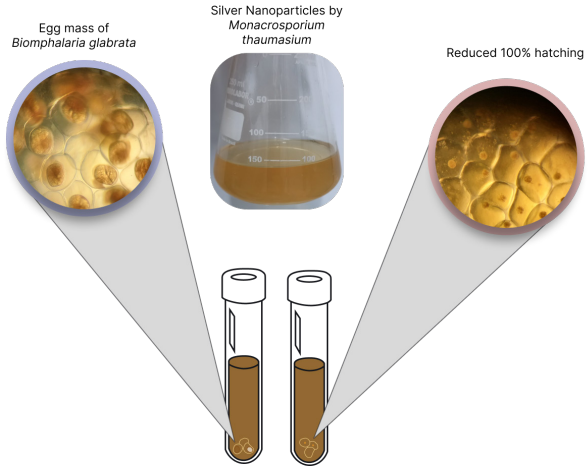
HIGHLIGHTS

- AgNPs exhibited potential molluscicidal activity against *Biomphalaria glabrata* snails, hindering hatching and the complete development of the organism's stages.
- Varied sizes of AgNPs demonstrated an ability to adhere and be absorbed by the snail surface, causing membrane permeability, cell death, and disrupting the life cycle of *B. glabrata*.
- *Monacrosporium thaumasium*, known for the predation of nematodes, demonstrated its capability to biosynthesize AgNPs.

GRAPHICAL ABSTRACT



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ABSTRACT

Nematophagous fungi are widely utilized for biological control against both helminths and their intermediate hosts. This study investigates the fungus *Monacrosporium thaumasium's* potential in synthesizing silver nanoparticles (AgNPs). The efficacy of a crude extract from an *M. thaumasium* isolate (strain NF34) combined with silver nitrate ($AgNO_3$) was tested on egg masses of *Biomphalaria glabrata*, serving as a model for embryotoxicity. The experiment followed a completely randomized design, with treatments containing AgNPs (in various proportions) and a control group with dechlorinated water maintained at 25 °C for ten days. Results indicate that *M. thaumasium* effectively produces AgNPs, causing 100% inhibition in exposed snail egg masses. The experimental results indicate that the fungus exhibits a potential molecular mechanism for nanoparticle formation, along with demonstrating embryotoxic activity in snail egg masses. These findings underscore the importance of further investigating this action and the underlying mechanism to provide potential applications in biological control.

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1. Introduction

Schistosomiasis is a public health problem and neglected tropical disease caused by *Schistosoma mansoni* [1]. This trematode has an intermediate freshwater-snail host, the *Biomphalaria* spp. In Brazil, the intermediate hosts are *Biomphalaria glabrata*, *B. tenagophila*, and *B. straminea*; it is estimated that 1.5 million people live close to endemic areas [2,3]. According to Medeiros *et al.* [4], these gastropods establish themselves in stagnant waters or lentic courses in neotropical areas. In general, to control these gastropods, it is necessary not only to treat the parasitized animals chemically but also to control the population density of the host molluscs [5]; draining flooded areas is not enough, as these organisms go into aestivation and can return [6].

Studies on biological control have highlighted nematophagous as promising in the control of gastrointestinal parasites (GIN) [7-11], while other studies have used gastropods [12,13]. Among biosystems, fungi have many advantages due to their high tolerance to heavy metals, ease of scaling, easy biomass handling, recovery, and economic viability [14].

The predatory fungus *Monacrosporium thaumasium* belongs to the helminthphagous or nematophagous fungi group and has mechanisms of action in the biological control of helminths [15]. In addition, nematophagous fungi, when in contact with helminth larvae or eggs, produce extra and intracellular substances that allow their predation mechanism [16]. Understanding its enzymatic characteristics, the use of predatory M. thaumasium's extracellular enzymes, particularly its viability to biosynthesis AgPNs for potential ovicidal activity, may be one more measure that can be applied to biological control.

It is known that most commercially used antimicrobial agents, including silver, are biocides for most microorganisms relevant to the medical field, including bacteria, fungi, and yeasts. The mode of action of these agents consists of damaging the cell wall or changing the permeability of the cell membrane, denaturing proteins, and inhibiting enzymatic activity or lipid synthesis, which are essential mechanisms for cell survival [17,18]. However, the reduction mechanism that forms silver nanoparticles (Ag^{+} to Ag^{0}) is still unknown [19] due to the fact that different biological agents react differently with metallic ions [20].

Several microorganisms involved in the biogenic production of nanoparticles, including bacteria, fungi, and yeast, are capable of synthesizing these compounds through their metabolism, that is, the secretion of extracellular enzymes [21]. When the synthesis occurs only in chemical and generally inorganic elements, three main components are needed: a silver salt (generally $AgNO_3$), a reducing agent responsible for the chemical transformation of the silver ion (generally sodium borohydride), and an agent to control and prevent its aggregation [22]. However, when the synthesis comes from microorganisms, the reducing agent and stabilizer are replaced by molecules produced by the organism itself [23]. In this regard, there are prominent predatory fungi, such as *Duddingtonia flagrans* [24], that can biosynthesize silver nanoparticles (AgNPs). Among these fungi, *Monacrosporium thaumasium*, a nematophagous fungus, has yet to elucidate its mechanism of biosynthesis of AgNPs in the *Biomphalaria* embryotoxic toxicity test (BET).

In this study, the biosynthesis of AgNPs mediated by the fungus *Monacroporium thaumasium* was performed and characterized by UV-Vis spectrophotometer, Dynamic light scattering, and Scanning electron microscopy. The resulting AgNPs were tested on egg masses of *Biomphalaria glabrata* to analyze their efficiency in controlling these snails.

2. Experimental

The fungus, *Monacrosporium thaumasium*, was provided by the Parasitology Laboratory, Department of Veterinary Medicine, Federal University of Viçosa, Brazil (DTV/UFV).

2.1. Culture media for the fungus

The fungus was cultured in PDA medium (Sigma-Aldrich) supplemented with 0.5% yeast extract and 2% malt extract and incubated in Biochemical Oxygen Demand (BOD) for five days at a controlled temperature (28 °C).

2.2. Synthesis of nanoparticles (AgNPs) mediated by the fungus Monacrosporium thaumasium

The synthesis of AgNPs was carried out in the Laboratory of Metabolism and Fermentation (LMF). Almeida *et al.'s* protocols were followed [25].

After a 5-day growth period for the fungal strain, 10 ± 0.5 g of NF34 isolate biomass scraped from the mycelium formed by the fungal strain on the PDA solid medium was transferred to an Erlenmeyer flask containing 100 ml of distilled water.

The sample was kept for 72 h at 28 °C in aqueous suspension in a horizontal incubator at 120 rpm in the dark.

After the 72 h, the biomass was separated from the medium by filtration using a quantitative paper filter with a porosity of 25 μm (12.5 cm). After filtration, 1 mM (10^{-3} M or 0.017 g/100 ml) of $AgNO_3$ (silver nitrate) was added to the fungal filtrate. Subsequently, the suspension containing the fungal filtrate with silver nitrate was kept for five days in an incubator in the dark at 28 °C in aqueous suspension in a horizontal incubator at 120 rpm.

After the biosynthesis processes, the suspension was stored at a temperature of 4 °C in the dark to prepare for the characterization of the reaction medium. The efficiency of the biosynthesis was evaluated by observing changes in the color tone of the reaction medium.

2.3. Dynamic light scattering (DLS)

Dynamic light scattering was used to measure the size of the silver nanoparticles. Briefly, about 2 ml of the colloidal samples containing the silver nanoparticles were first transferred to a test tube. Then, the component containing the laser light source was immersed in the sample. The equipment's software analyzed the frequency of light incident on the particles, generating a histogram containing the percentages of the different sizes of the nanoparticles. The equipment used was a multiangle light scattering Brookhaven Co. with a laser He-Ne, 632.8 nm, performed at the Laboratory Microfluidic and Complex Fluid (DPF/UFV).

2.4. Characterization via spectroscopy in the visible ultraviolet region (UV-Vis)

The aliquots containing the nanoparticles were periodically removed and subjected to UV-Vis spectroscopy analysis using a spectrophotometer (Shimadzu UV–Vis-1501). Fluorescence emission measurements were performed using a 378 nm Coherent Cube Laser as an excitation source and an Ocean Optics USB4000 spectrometer to collect the signal from all samples and references. To do so, about 2 ml of samples containing colloidal silver nanoparticles were analyzed in quartz cuvettes. All UV-Vis analyses were performed at the Sample Preparation Laboratory (Production of Semiconductor Nanostructures and Nanocomposites) - UFV.

2.5. Scanning Electron Microscopy (SEM) / Energy Dispersive Spectroscopy (EDS) Analysis

The shape and spatial aspects of the silver nanoparticles were characterized by Scanning electron microscopy (SEM) acquired by a JEOL JSM-6010/LA microscope equipped with an Energy dispersive spectrometry system (EDS) to verify the chemical composition of the silver nanoparticles. SEM images and EDS spectra were obtained with a working distance of 10 mm and an accelerating voltage of 16 kV. All analyses were performed in the Scanning Electron Microscopy Laboratory - (DPF/UFV). Samples of silver nanoparticles were prepared by dropping them onto a small, previously cleaned, and sterilized aluminum stub. The stubs were then placed in a desiccator containing silica gel, where they remained for 48 h to promote sample drying.

2.6. Obtaining egg masses of Biomphalaria glabrata

The snails used in this study were obtained from the Laboratory of Veterinary Helminthology at the Institute of Biological Sciences of the Federal University of Minas Gerais (UFMG). Generations already kept under laboratory conditions were placed in glass aquariums in dechlorinated water with artificial aeration, temperature, and ambient photoperiod. Such organisms were fed lettuce leaves *(Lactuca sativa) ad libitum*. To obtain the egg masses (Fig. 1), polystyrene plates (± 4 cm^2) were placed inside the aquariums to serve as substrates for oviposition.

2.7. Biomphalaria embryotoxic test (BET)

The egg masses were gently collected with the aid of a sterile sowing loop and transferred to a glass plate with dechlorinated water (new water, but in the same condition as the water in the aquarium where the adult snails are found) in ratios of 0.5 ml×1 ml, 2 ml×0.5 ml, and 1 ml×1 ml. They then built three test tubes containing dechlorinated water and egg masses in the above proportions for the control group and three more for the treatment group with dechlorinated water, egg masses, and AgNPs in the same proportions.

The experiment was arranged in a completely randomized design, consisting of three control groups (C1, C2, and C3 of dechlorinated water and egg mass) and three treatment groups (dechlorinated water, egg mass, and AgNPs) in triplicate and maintained in the same ambient, light, and temperature conditions. The results were expressed as mean ± standard deviation. One-way ANOVA tests were conducted with one factor: hatchability x treatments, and Minitab software (version 18, Minitab Corporation) was used to compare means.

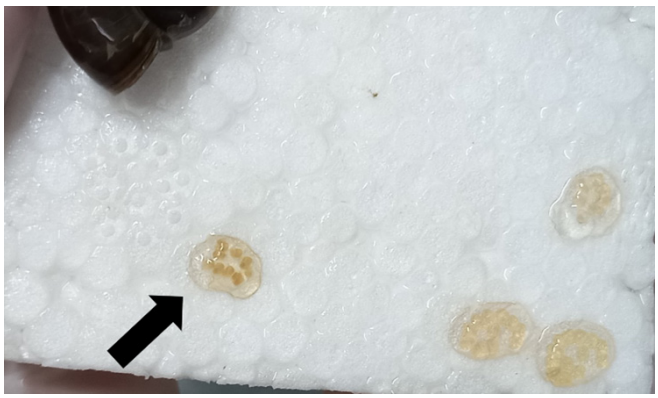

Fig. 1. *Biomphalaria glabrata* egg masses deposited on the polystyrene plate.

3. Results and disscusion

The fungus *M. thaumasium* was grown in PDA, malt extract, and yeast extract, and after five days of incubation, it was possible to see the growth of the mycelium (Fig. 2) required to proceed to the stages of biosynthesis of silver nanoparticles (AgNPs).

Subsequently, the mycelia obtained from cultivation in PDA were weighed and scraped lightly with scalpel blades (stainless steel) in a sterile environment and inoculated in 250 ml Erlenmeyer flasks containing 100 ml of ultrapure water (Fig. 3). The flasks were again kept in the same temperature conditions and incubated for 72 h to allow new growth in the liquid medium.

After the biosynthesis of AgNPs, the nanoparticles were subjected to a partial determination of the characteristics of this suspension, which would later be used as an agent in BET. The biosynthesis of AgNPs was promoted by adding silver salt ($AgNO_3$) to the fungal filtrate, thus allowing proteins and other biomolecules biosynthesized by *Monacrosporium thaumasium* to act in the bioreduction mechanism of silver ions as well as in the stabilization process of nanoparticles.

With regard to the efficiency of fungus-mediated biosynthesis, a change in the color of the reaction medium (Fig. 4a) was evident when compared to the fungal filtrate (Fig. 4b). So, the fungus secretes an extracellular enzyme that will probably promote the reduction of silver ions, resulting in the formation of metallic silver nanoparticles.

The UV-Vis spectra were recorded from samples in cuvettes containing the fungal filtrate with and without the addition of $AgNO_3$ (1 mM) and then subjected to optical measurements using a UV-Vis spectrophotometer. The analysis showed that the surface resonance peak is strongly accentuated at around 500 nm (Fig. 5). This result is close to biosynthesis by Duddingtonia flagrans [24], another fungus with nematophagous activity.

Light emission was observed in only the NF34 sample with water, which may be due to the fungi containing common fluorophores, including NAD(P)H, flavin derivatives, flavoproteins, and lipofuscins. Moreover, the pigment melanin can affect the autofluorescence signal as an internal absorbing filter [26].

In Fig. 6, SEM images show the morphology of AgNPS, indicating that biosynthesis produces different aggregate sizes and dispersed forms [27]. Using the EDS spectra in Fig. 6(c), we were able to confirm chemical elements in the sample, which established the presence of silver.

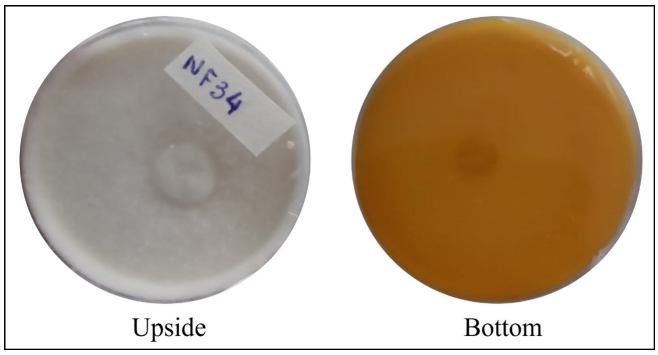

Fig. 2. Culture plate containing the fungus *Monacrosporium thaumasium* cultivated in PDA culture medium, malt extract, and yeast extract at 28 °C after five days incubated in the dark.

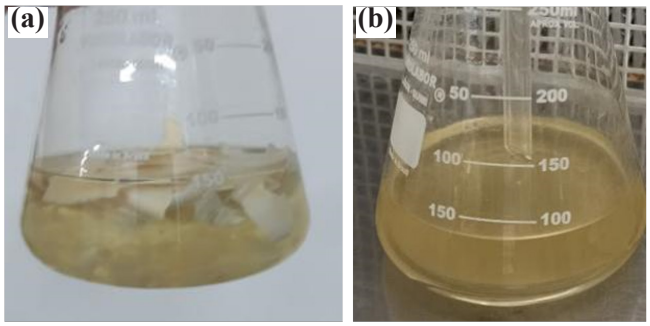

Fig. 3. (a) Flask containing 10 g of the fungal mycelium of *Monacroporium thaumasium* in 100 ml of ultrapure distilled water. (b) Flask containing fungal filtrate of *Monacrosporium thaumasium* after 72 h of incubation at 28 °C in the dark.

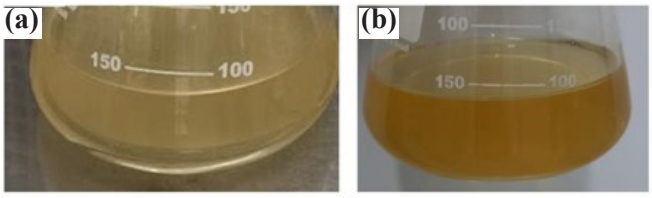

Fig. 4. Reactional alteration of suspensions obtained through the fungus *Monacrosporium thaumasium*. (a) Fungal filtrate of NF34 with distilled water. (b) Fungal filtrate with silver nitrate ($AgNO_3$).

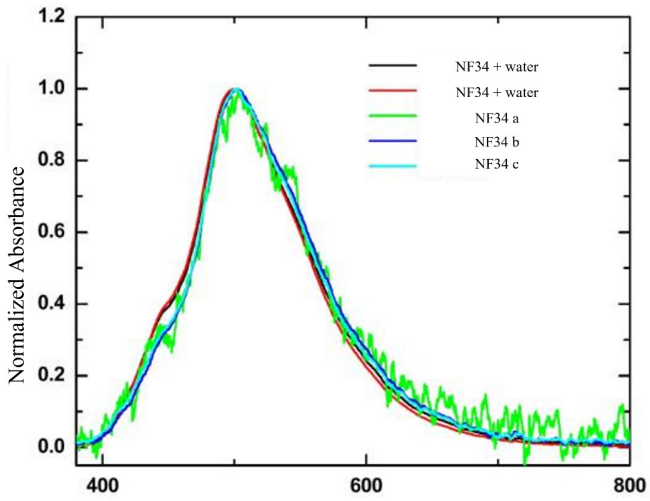

Fig. 5. Recorded normalized UV-visible spectra of biosynthesis of silver nanoparticles mediated by *Monacrosporium thaumasium*.

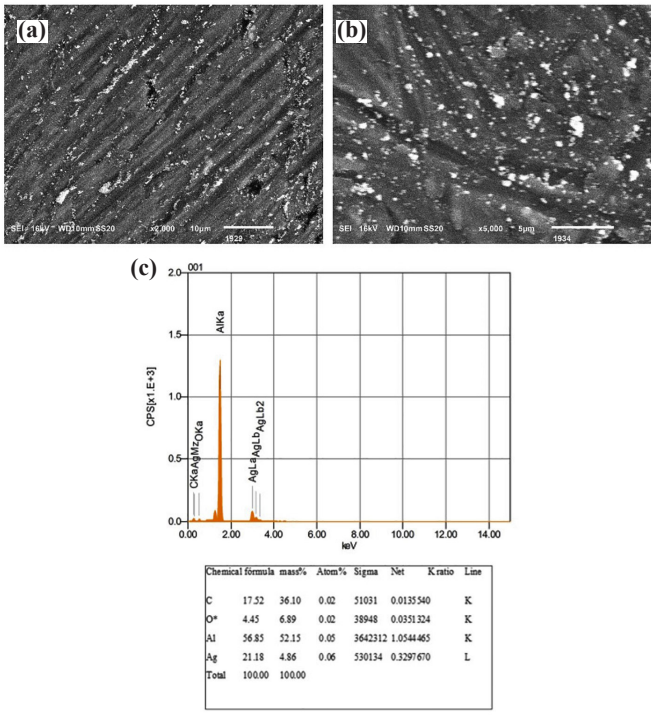

Fig. 6. SEM–EDS Micrograph of silver nanoparticles biosynthesized using the fungus *Monacrosporium thaumasium*: (a) 2000x magnification; (b) 5000x magnification; and (c) Histogram of inorganic and organic components of the AgNPs sample.

Dynamic Light Scattering (DLS) is a technique that studies the correlation of light intensity scattered by the sample after it is hit by an electromagnetic wave. The hydrodynamic radius can be obtained, assuming the particles have a spherical shape according to the Stokes-Einstein relationship. The equipment provides the autocorrelation function, $g^{(2)}(\tau)$ - 1. Measurements were performed for the detection angles 30°, 40°, 60°, and 90° as shown in Fig. 7.

The results from Fig. 7 were analyzed using Eq. (1). As the autocorrelation functions were adjusted, two populations of the structures with different sizes were noticed in the solution.

$$g^2(\tau) - 1 = [\beta_1 \exp(-\Gamma_1 \tau) + \beta_2 \exp(-\Gamma_2 \tau)]^2 \quad (1)$$

By fitting Eq. (1), we obtain the Γ_1 and Γ_2 for the two populations related to the decay rate for the two exponential equations. The Γ could relate to the diffusion coefficient D and the wave vector q through Eq. (2). The coefficients β_1 and β_2 were related to the exponential amplitude.

$$\Gamma = D\, q^2 \quad (2)$$

As seen in the graph of Γ vs. q^2 in Fig. 8, it is possible to obtain the diffusion coefficient through a linear fit using Eq. (2). The obtained diffusion coefficients and the hydrodynamic radius values are shown in the experiment conditions: η = 0.891 cP, n = 1.331, and λ = 632.8 nm.

Therefore, this particle has an average hydrodynamic radius of 156.45 nm. It was observed that the particles do not have a completely spherical shape but rather a small elongation. However, this elongation was small, and the emitted signal had low intensity and did not favor the characterization, making it possible to make measurements for low scattering angles like 30° (Fig. 9).

This measurement was performed using the polarizer in the horizontal position, so there will only be a signal if the particles are not perfectly spherical.

After the characterizations, AgNP toxicity tests were carried out in the Biomphalaria glabrata egg masses. The suspension containing AgNPs promoted a hatching inhibition rate of

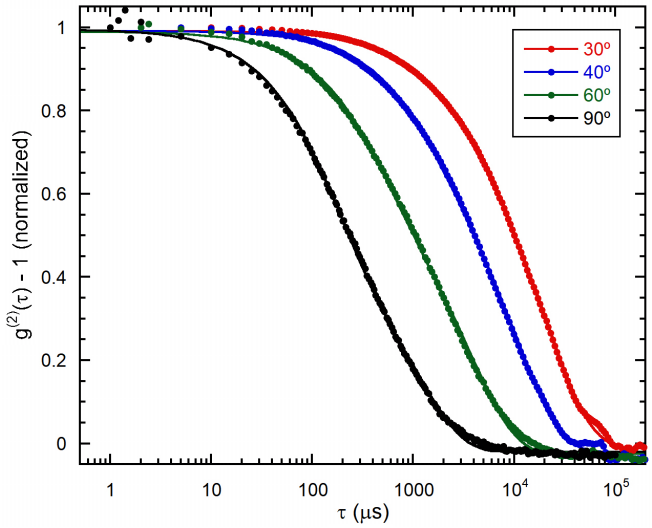

Fig. 7. Sample autocorrelation function for the 30°, 40°, 60°, and 90° scattering angles of the reaction medium obtained through the biosynthesis of silver nanoparticles (AgNPs) by the fungus *Monacrosporium thaumasium*. All the curves were fitted by Eq. (1).

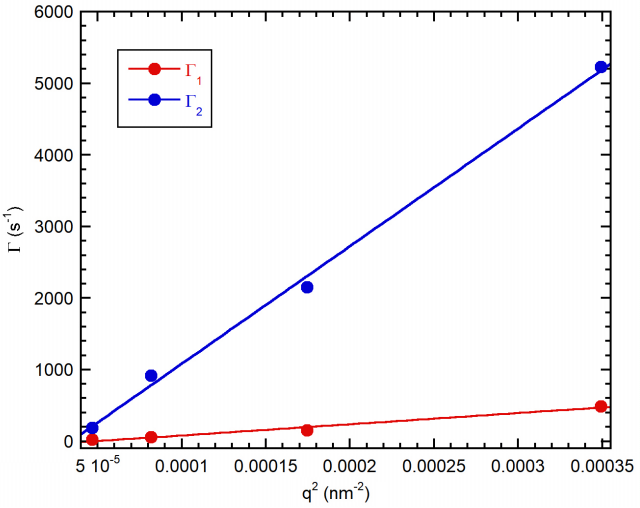

Fig. 8. Graph of Γ using the squared scattering vector for the scattering angles of 30°, 40°, 60°, and 90° of the reaction medium obtained through the biosynthesis of silver nanoparticles (AgNPs) by the fungus *Monacrosporium thaumasium*. The lines are linear adjustments to obtain the diffusion coefficient.

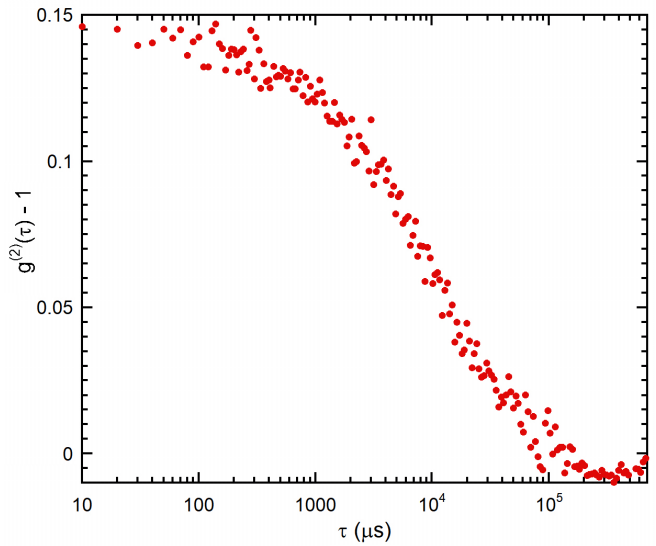

Fig. 9. Autocorrelation function for a scattering angle of 30° and with the polarizer positioned horizontally.

100% (Fig. 10) in the snails during the 10 days of exposure.

It can be observed that all treatments containing the AgNP solution were sensitive to the test compared to the control group (Fig. 11).

The biosynthesis of AgNPs was promoted by adding silver salt ($AgNO_3$) to the fungal filtrate, thus allowing proteins and other biomolecules biosynthesized by *Monacrosporium thaumasium* to act in the bioreduction mechanism of silver ions as well as in the stabilization process of nanoparticles.

Evidence of the formation of AgNPs, as demonstrated after the addition of silver nitrate (1 mM) to the flask containing the fungal filtrate, is demonstrated by the change in color of the medium, which changed to a yellowish-brown hue 5 days after the addition of the salt silver. The appearance of this color suggests the formation of silver nanoparticles in solution [28,29]. Studies involving other fungi by Basavaraja

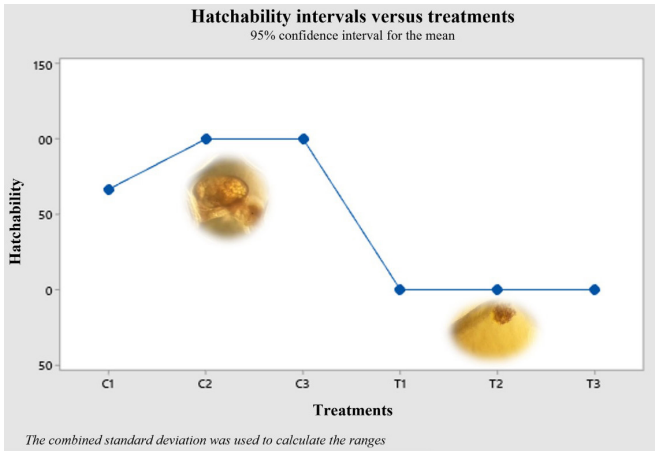

Fig. 10. Snail hatchability rate. C1, C2, and C3 – Control group with dechlorinated water and egg masses (not exposed to silver nanoparticles). T1, T2, and T3 – Exposure treatment with AgNPs in egg masses of *Biomphalaria glabrata* for ten days at 25 °C.

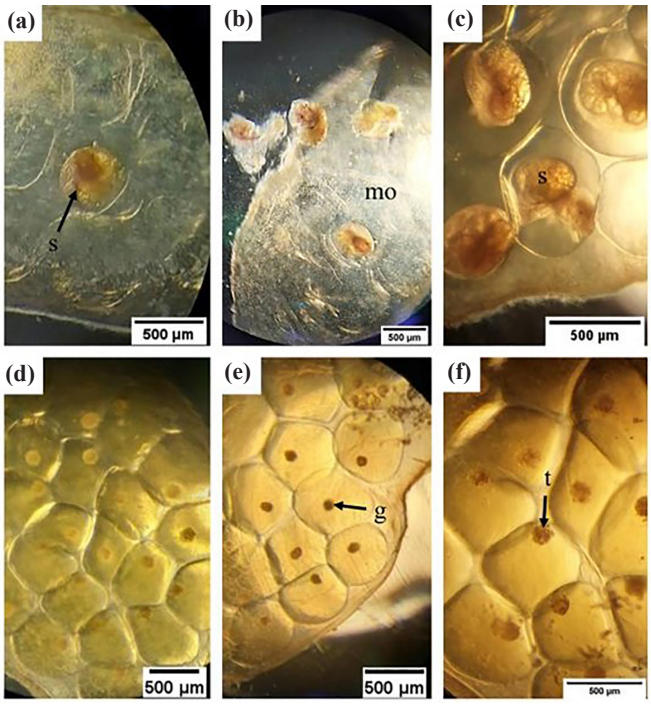

Fig. 11. External aspect of changes in *Biomphalaria glabrata* egg masses. (a, b, and c) Control group-dechlorinated water; (a)-s-snail (snail); (b, c) mo–egg mass; (d, e, and f) Treated group– (d) dechlorinated water and (e, f)-AgNPs; and f) Stage of the snail in the trochophore phase (t). Scale = 500 μm.

et al., verified that the color change occurs due to the reduction of Ag^+ ions to Ag^0 according to the release of extracellular enzymes [30]. The findings of this experiment are similar to the results found by Oliveira-Filho *et al.* [31], in which a rapid accumulation of the AgNPs suspension was observed and interfered with the reproduction of these snails. Tests with a 100 $\mu g.ml^{-1}$ concentration of AgNPs also obtained a 100% lethal effect on snails [32].

The intensity of the UV-Vis peak was directly proportional to the yield of NP biosynthesis; thus, although it is still not fully understood, the current most accepted theory for the exact mechanism of AgNPs biosynthesis implies that the silver ions generated by the addition of $AgNO_3$ to the fungal filtrate require the enzyme nitrate reductase dependent on NADPH for its reduction (Ag^+ to Ag^0) [33]. Given this, the enzymatic complex responsible for the bioreduction of silver ions was synthesized and released by the fungus *Monacrosporium thaumasium*.

The results of this study are satisfactory in suggesting treatments based on AgNPs in egg masses since neither the hatching of any snail nor the complete development of the stages of this organism was observed. The varied sizes of AgNPs demonstrated a potential ability to adhere to and be absorbed by the surface of target agents (snails) and induced the mortality of these snails, which are intermediate hosts of schistosomiasis. As a result, the membrane of these masses

suffered permeability, causing cell death and revealing that AgNPs functioned as molluscicidal activity against *B. glabrata*.

It is understood that the fungus *M. thaumasium* acts as a predator of nematodes through adhesive networks [34]. However, the use of extracellular enzymes from fungi and other microorganisms act at different stages that interfere with the development of larvae or hatchability [35,36]. Among the fungi used as biological controls, the D*uddingtonia flagrans* isolate was also used as NPs and tested on nematodes, showing promising results as a biological control [24].

According to Almeida *et al.*, the amount of biomass plays a key role in the mechanism of bioreduction of Ag^{+} ions to Ag^{0} [25]. An increase in the amount of biomass in the reaction medium allows a higher release of reductase enzymes and other proteins responsible both for the bioreduction process and also for the stabilization process of silver nanoparticles. Therefore, in this present work, these enzymes were found to be a suppressor and toxic agent in the egg masses and, consequently, in the eggs, due to the Ag ions. However, the exact characterization mechanisms of these enzymes and their toxicity require further studies.

4. Conclusion

The study demonstrates the potential of utilizing the nematophagous fungus *Monacrosporium thaumasium* in synthesizing silver nanoparticles (AgNPs) for biological control purposes. By combining a crude extract from *M. thaumasium* with silver nitrate ($AgNO_3$), the experiment effectively inhibited egg masses of *Biomphalaria glabrata*, a model for embryotoxicity with 100% effectiveness. This suggests a promising avenue for utilizing *M. thaumasium*-derived AgNPs in combating both helminths and their intermediate hosts. Moreover, the study hints at a potential molecular mechanism underlying nanoparticle formation by the fungus, highlighting the need for further research to elucidate this process and explore its applications in biological control strategies.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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