Susceptibility of embryos of *Biomphalaria tenagophila* (Mollusca: Gastropoda) to infection by *Pochonia chlamydosporia* (Ascomycota: Sordariomycetes)

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Abstract

*Schistosoma mansoni* is a heteroxenous parasite, meaning that during its life cycle needs the participation of obligatory intermediate and definitive hosts. The larval development occurs in aquatic molluscs belonging to the *Biomphalaria* genus, leading to the formation of cercariae, which emerge to infect the final vertebrate host. For this reason, studies for control of the diseases caused by digenetic trematodes often focus on combating the snail hosts. Thus, the objective of this study was to evaluate the susceptibility of *Biomphalaria tenagophila* embryos to the fungus *Pochonia chlamydosporia* (isolate Pc-10). The entire experiment was conducted in duplicate, with five replicates for each repetition (five egg masses/replicate), utilizing a total of 100 egg masses, with 20–30 eggs/egg mass. At the end of 15 days, the egg masses were evaluated under a stereomicroscope to analyze the hatching of *B. tenagophila* embryos in both experimental groups. After days of interaction, the exposure to the fungal hyphae bodies significantly impaired the viability of the *B. tenagophila* eggs, inhibiting the embryogenesis process by 83.7% in relation to the control group. Transmission and scanning electron microscopic images revealed relevant structural alterations in the egg masses exposed to the hyphae action of the fungus, interfering in the development and hatching of the young snails under analysis. These results indicate the susceptibility of *B. tenagophila* embryos to the fungus *P. chlamydosporia* (isolate Pc-10) and suggest the potential of Pc-10 to be used in the control of intermediate host, for its ovicidal capacity and for being an ecologically viable option, but in vivo experiments become necessary.

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Introduction

Mansonic schistosomiasis is endemic in Brazil, showing as etiological agent the trematode *Schistosoma mansoni* (Brasil 2010). This disease of public health relevance has an acute and/or chronic evolution, where the clinical manifestations range from mild dermatitis to relevant hepatosplenic alterations, characterized by hepatopathies with periporal fibrosis, portal hypertension, splenomegaly, and ascites (Bezerra et al. 2004; Lamberton et al. 2014). According to epidemiological reports from official agencies, schistosomiasis affects more than 200 million people in the world annually (WHO 2011), of which 1.5 million cases occur in Brazil (Brasil 2019).

Various factors contribute to the establishment and dispersal of mansonic schistosomiasis in Brazil, such as the widespread presence of the snails that act as intermediate hosts of the helminth (Gastropoda: Planorbidae); the movement (temporary or permanent) of people from endemic areas; deficient residential and environmental sanitation; and lack of health education of people at risk of infection (Passos and Amaral 1998; Nacife et al. 2018). Therefore, the control of this parasitosis requires the adoption of measures including effective drug treatment of infected definitive hosts, implementation of public policies to improve sanitary conditions in affected regions, sanitary education of target publics, and population control of the snails that integrate the disease’s epidemiological transmission chain (WHO 2011).

Snails of the *Biomphalaria* genus are hermaphrodites, being capable of self- or cross-fertilization; the eggs are laid at batch intervals of 5–40 units, each batch being covered in a mass of gelatinous material, called an egg mass. Young snails hatch after 6–8 days and reach maturity in 4–7 weeks, depending upon the species and environmental conditions (WHO 1997). According to Paraense (1972), in the presence of temporary aquatic environments, such as training of puddles that gradually reduce, these snails carry out the behavior of burying themselves in the ground, thus assuming amphibious behavior.

According to the United Nations (UN), among molluscicidal substances, a standout is niclosamide (5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide) (Machado 1982; Cantanhede et al. 2010) as well as copper sulfate.
(CuSO₄) and calcium cyanamide (CaCN₂). Although effective as a molluscicide, these substances are extremely toxic to the environment and human and animal health (Henrioud 2011), so it is not considered safe. Additionally, the use of these molecules for extended periods favored the selection of resistant snails, making its use in official control programs untenable (Andrews et al. 1982), condition that reinforces the importance of studies aimed at the development of new technologies related to the control of these hosts.

This situation has encouraged the development of alternative control methods, targeting the host snail, involving application of biodegradable substances obtained from plants (Hartmann et al. 2011), alone or in association with pathogenic organisms such as entomopathogenic nematodes (Tunholi et al. 2017a, b), bacteria (Cheng 1986; Singer et al. 1997), and fungi (Duarte et al. 2015; Castro et al. 2019). Fungi are widely found in nature and can trigger infectious processes in many organisms, thus acting as natural pathogens. The use of fungi to control pests through bioproducts is already a reality in agriculture, being produced on a commercial scale, to demand this purpose (Chagas et al. 2016). However, little is known about the pathogenicity of fungi against B. tenagophila embryos.

Evidence about the susceptibility of B. glabrata to aquatic and terrestrial pathogenic fungi has been reported by some researchers (Rocha et al. 2009; Baron et al. 2013). Duarte et al. (2015) observed impairment of the viability of B. glabrata eggs when exposed to the action of conidia and hyphae bodies of Metarhizium anisopliae. Furthermore, Castro et al. (2019) investigated the ovicidal potential of Pochonia chlamydosporia (isolate Pc-10) on eggs of Pseudosuccinea columella. According to them, P. chlamydosporia (Pc-10) induced severe structural alterations in the egg masses of the limned in question, hampering the development and hatching rate of the snails. The results of both studies indicated the possible use of these fungal species in programs for biological control of the snails. The susceptibility of B. tenagophila embryos to the fungus P. chlamydosporia has not been described yet.

The objective of this study was therefore to evaluate the susceptibility of B. tenagophila embryos to the fungus P. chlamydosporia (isolate Pc-10), as possible tool to be used in control of schistosomiasis intermediary hosts.

Materials and methods

Obtaining and cultivating the fungus Pochonia chlamydosporia (Pc-10)

The isolate Pc-10 of the fungus P. chlamydosporia used was obtained from the company Rizotec® (Rizotec®), located in the city of Viçosa, Minas Gerais, Brazil. The bioproduct is a microbiological nematicide, effective in combating nematodes, especially Meloidogyne. The isolate (powder formulation) was cultured in medium-containing potato dextrose agar (PDA) for 7 days in a biochemical oxygen demand (BOD) incubator at 27 °C and 80% relative humidity (RH) (Castro et al. 2019).

Obtaining egg masses of Biomphalaria tenagophila

Specimens of B. tenagophila were collected from cattle water troughs and ponds located in the municipality of Alegre (20° 45′ 48″ South, 41° 32′ 2″ West), Espírito Santo, Brazil. The adult snails (n = 180), having between 32 and 35 mm of shell diameter, were taken to the Parasitology Laboratory of the Veterinary Hospital of the Centre for Agrarian and Engineering Sciences of Federal University of Espírito Santo (CCAE-UFES), where they were maintained at average temperature of 24 °C in five glass aquariums containing dechlorinated water with continued mechanical aeration. The snails were fed with lettuce leaves (Lactuca sativa) ad libitum. The aquariums were cleaned weekly, and the lettuce leaves were replenished on alternate days to prevent their fermentation. Polystyrene dishes (± 5 cm²) were placed inside the aquariums to act as substrate for oviposition and to obtain the egg masses.

The egg masses used in this study were obtained from generations of a colony of B. tenagophila maintained in the laboratory, thus confirming the absence of infections. A total of 100 egg masses were obtained after 24 and 72 h of their respective postures for laboratory analysis. These were removed from the laying substrate with the support of a sterile stainless-steel spatula. They were examined under a stereomicroscope to confirm the presence of eggs. Only the egg masses containing viable embryos, undergoing development, were selected for the experimental testing. The egg masses, each containing about 20–30 eggs, were washed three times in distilled water and placed in sterile conical centrifuge tubes (50 mL) containing distilled water at room temperature until the start of the tests.

Exposure of the egg masses of Biomphalaria tenagophila to the fungus Pochonia chlamydosporia

Before the start of each test, the viability of the chlamydospore (> 95%) was checked according to the method described by Duarte et al. (2015). The biological product (Rizotec®) used in the execution of the study had a concentration of 5.2 × 10⁷ chlamydospores/g of P. chlamydosporia. Petri dishes (60 × 15 mm) with a permanent layer of water on 2% water-agar medium were used for the experimental exposure of the egg masses (n = 5) to the hyphae bodies of the fungus, containing approximately 72 × 10⁵ chlamydospores/mL of fungus. These dishes were placed...
in the center of larger dishes (80 × 15 mm), and filled with 2 mL of distilled water to keep the humidity and other environmental conditions favorable to the snails and growth of the fungus (Castro et al. 2019). Then, the larger Petri dishes (80 × 15 mm) were sealed with Parafilm® to maintain the relative humidity. These dishes were incubated in the dark at 23 ± 2 °C for 15 days. This period, in this temperature condition, ensures the completion of stages of gastrulation, organogenesis, and development of germ cells that are part of the embryogenesis process of B. tenagophila (Kawazoe 1976).

Two experimental groups were formed, the control group without exposure to the fungus, and the treated group, with exposure of the egg masses to the fungal isolate Pc-10. The entire experiment was conducted in duplicate, with five replicates for each repetition (five egg masses/replicate), using a total of 100 egg masses and between 2000 and 3000 viable eggs.

The evaluation of the interaction between egg masses of P. columella and hyphae bodies of P. chlamydosporia (in types 1, 2, and 3) was carried out according to the parameters defined by Lýsek and Stěrba (1991).

**Scanning and transmission electron microscopic (SEM and TEM)**

After incubation for 15 days, the egg masses (n = 3 egg masses/group) of both experimental groups (control and treated) inserted in microtubes (1.5 mL) containing a 2.5% glutaraldehyde solution (Castro et al. 2019). The samples were processed at the Carlos Alberto Redins Laboratory of Cell Ultrastructure (LUCCAR) of Federal University of Espírito Santo (UFES), Brazil.

The samples for SEM analysis were dehydrated in serial solutions of ethyl alcohol at concentrations of 30%, 50%, 70%, 90%, and 100% for 10 min at each concentration. Then, each sample was dried to critical point in a TOUSIMIS, Autosamdr®-815 apparatus using CO₂, the samples were attached to a metal support with double-sided carbon tape and coated with a layer of gold of approximately 250 Å, using a Denton Vacuum Desk V system. The samples were observed under a scanning electron microscope (JEOL-JEM-6610, USA).

For TEM, the samples were dehydrated by passage in a rising serial solution of acetone (50%, 70%, 90%, and 100%), with gradual inclusion in increasing proportions of Epon:acetone, embedded in pure Epon epoxy resin, and placed in an oven at 60 °C for 24 h for polymerization. After the polymerization, the blocks containing the samples were cut into ultra-thin sections (60 nm) with an RMC Products Power Tome X ultramicrotome with glass face, collected on copper grids (400 mesh), and contrasted with 0.5% uranyl acetate and lead citrate (Machado and Souza 1998) for visualization of the biological structures (egg masses and fungi) using a transmission electron microscope (JEOL-JEM 1400, USA).

**Hatching rate of Biomphalaria tenagophila exposed to the mycelial activity of Pochonia chlamydosporia**

After incubation for 15 days, the egg masses of B. tenagophila exposed and not exposed to the fungus were analyzed with a stereomicroscope to count the number of hatched snails. The viability of the eggs was measured according to the percentage of hatched snails in relation to the total number of eggs laid by the snails in each experimental group during the experiment.

**Statistical analyses**

The study was carried out in a completely randomized design (CRD) and the Shapiro–Wilk test was applied to confirm the normal distribution of data. The results were expressed as mean ± standard deviation and subjected to Student’s t test, followed by one-way analysis of variance (ANOVA) and the Tukey–Kramer test (p < 0.001) to compare the means (GraphPad Prism Inc. 6.01, R 3.4.1).

**Results**

After incubation for 15 days, the exposure to the hyphae bodies of P. chlamydosporia (isolate Pc-10) impaired the embryogenesis process of B. tenagophila (p < 0.05). While the eggs of the control group had a hatching rate of 98% (93.95 ± 2.75), the eggs exposed to the mycelial action of the fungus had a hatching rate of only 14.3% (13.70 ± 0.78) (Fig. 1), demonstrating a proportional efficacy of 83.7% of the Pc-10 isolate in interfering with the embryogenesis process of B. tenagophila.

![Fig. 1](image-url) Hatching rate of Biomphalaria tenagophila exposed (treated) and not exposed (control) to propagules of the fungus Pochonia chlamydosporia isolate Pc-10. ***Averages differ significantly each other (mean ± SD). P < 0.001
Based on the observations by scanning and transmission electron microscopy, two types of interaction were noted: type 1 (Fig. 2), characterized by a physiological and/or biochemical effect, exerted here by the chemical action of toxins and enzymes arising from the process of metabolism of the fungus that interfere in the stages of embryogenesis of eggs, without affecting the morphology of the egg shell, where hyphae were observed adhered to the egg mass surface; and type 2 (Fig. 3), indicated by a lithic effect with alteration of the egg shell morphology and impairment of the embryonic development, without hyphal penetration of the egg shell. Type 3 interaction, involving a lithic effect accompanied by altered morphology of the embryo and egg, optimized by penetration of hyphae and internal colonization of the egg was not observed during the period studied.

Additionally, the images of TEM showed part of the thick mass of eggs, ruptured (Fig. 4A, B), and it was also possible to visualize the structure of the chlamydospore and prolongation of the hyphae (Fig. 4C). In the SEM (Fig. 4D–F), it was possible to visualize the ruptured mass of the control group, being the inner part without the presence of hyphae (Fig. 4E). Different from the egg mass that was treated with the fungus (Fig. 4G–I), where there is the presence of mycelium and hyphae, along with the presence of chlamydospores that colonized the interior.

Melanization was not observed in the egg masses exposed to the fungus (Fig. 5B) from the 15th day of incubation. No fungal growth or melanization occurred in the control group (Fig. 5A).

**Discussion**

Unfortunately, many control programs for mollusc species that host parasites of human and animal species, whether of economic interest or of companionship, let us look at the example of *Biomphalaria*, do not have achieved success. The scarcity of chemical molecules with molluscicide potential associated with the ban, by official bodies, of the use of these compounds in the environment contributes in part to this. The present study is the first, under laboratory conditions, the to analyse the susceptibility of *B. tenagophila* embryos to the fungus *P. chlamydosporia*.

In the present study, the experimental exposure to the hyphae bodies of *P. chlamydosporia* (Pc-10) inhibited the hatching rate of *B. tenagophila* by 83.7%. In general, fungi are biological agents found naturally in the soil that are pathogenic to various species of invertebrates (Rocha et al. 2009; Baron et al. 2013). Among the fungal species utilized as biocontrol agents, *P. chlamydosporia* stands out. It is a member of the Ascomycota phylum with global distribution, generally isolated from soils rich in organic matter (Manzanilla-Lopez et al. 2013). It is classified as a facultative parasite of the eggs of molluscs and helminths, as well as a hyperparasite of other types of fungi. For these reasons, it has been extensively used for control of many parasitoses (Zare et al. 2001; Braga and Araújo 2014).

Braga et al. (2008) evaluated in vitro the effect of *P. chlamydosporia* against *S. mansoni* eggs and found that the exposure to the fungus significantly impaired the egg viability. According to Escudero et al. (2016), the ovicidal potential of Ascomycota fungi results from the mechanical action imposed by their hyphae bodies during the germination step, associated with the release of exoenzymes such as, chitinases and proteases, which compromise the egg tegument, directly interfering in the establishment of the embryo.
Among the proteases, the serine–alkaline protease VCP1, stands out by degrading the protein layer that composes the external membrane of helminth eggs (Braga et al. 2011). In this respect, the interruption of the embryogenesis process in *B. tenagophila* verified in this study might have been the result of similar interactions developed by hyphae of *P. chlamydosporia* (isolate Pc-10) when adhering to the surface of the egg masses of this planorbid, interfering with about 84% of the hatchability rate of *B. tenagophila* embryos. These results demonstrate the ovicidal capacity of *P. chlamydosporia* on *B. tenagophila* egg masses, proving to be a promising alternative in the control of schistosomiasis.

Recently, the embryotoxic effect of *P. chlamydosporia* against *Pseudosuccinea columella* was described (Castro et al. 2019). Through scanning and transmission electron microscopies analysis, the authors observed that mycelial
growth provoked important alterations in the membranes covering the embryos, contributing to loss of water, electrolytes, carbohydrates (e.g., galactogen), and other nutrients that are essential to the embryonic development of the gastropod (Goudsmit 1972; Faro et al. 2013). Additionally, Castro et al. (2019) observed that the egg masses exposed to the fungus had a withered aspect, with deep grooves on the surface, probably caused by the nutritional demands of the fungus. This alteration contributed to the 93.15% inhibition of embryogenesis of the host, suggesting the applicability of the Pc-10 isolate in biological control programs. Analogous mechanisms to those possibly occurred in the present study, explaining in part the 83.7% inhibition of the viability of B. tenagophila eggs.

Assessing the effects caused by ovicidal fungi, such as P. chlamydosporia, Frassy et al. (2010) observed that only the type-3 effect is considered embryotoxic, capable of compromising the development of embryos from target nematode populations. On the other hand, in the present study, it was possible to observe that type 1 and 2 effects were also able to significantly interfere in the viability of B. tenagophila eggs, suggesting the embryotoxic potential of these effects in B. tenagophila eggs.

Duarte et al. (2015) found that the viability of eggs and the maturation of egg masses of B. glabrata diminished significantly after exposure to the conidia and hyphae of M. anisopliae. According to the authors, the inhibition of embryogenesis possibly occurred through secretion/excretion of substances resulting from the fungal metabolism, which disseminated in the egg masses and impaired the development of embryos. Associated with this, Przeslawski and Benkendorff (2005) reported evidence that the reduction of the oxygen content inside egg masses of gastropod molluscs is a factor limiting egg viability. Therefore, the mycelial activity of P. chlamydosporia (isolate Pc-10) on egg masses of B. tenagophila might have caused a deficit of oxygen available to the embryos as well as the release of embryotoxic substances, hindering hatching.

Castro et al. (2019) also reported the occurrence of melanisation of snail egg masses in response to exposure to fungal propagules. Melanin is a hydrophobic and negatively charged pigment, synthesized by oxidative polymerization of phenolic compounds. Its production is a defense mechanism against pathogens that is very important for various invertebrates, including Biomphalaria (Bai et al. 1996; Bahgat et al. 2002). Nevertheless, we did not observe this alteration. The results obtained show promise characterizing the ovicidal potential of P. chlamydosporia in B. tenagophila.

**Conclusion**

This study reports for the first time the susceptibility of B. tenagophila embryos to infection by P. chlamydosporia (isolate Pc-10). The proposed method is significant impairing the hatchability of the snail, which is an intermediate host of S. mansoni. Therefore, the biological mechanism of the fungus can be considered an attractive alternative for control of this intermediate host population density.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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