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ARTICLE

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ENZYMATIC ACTIVITY OF THE FUNGUS *Pochonia chlamydosporia* (SORDARIOMYCETES: HYPOCREALES) AND ITS OVICIDAL POTENTIAL ON EGGS OF *Toxocara canis* (NEMATODA: ASCARIDIDA)

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Abstract

Biological control is considered one of the most used alternative measures to combat helminths of relevance in veterinary medicine and public health. Among these parasites, *Toxocara canis* stands out for its high prevalence and worldwide distribution, in addition to being the main cause of visceral larva *migrans* in man. The present work aimed to demonstrate the ovicidal activity of the fungus *Pochonia chlamydosporia* (isolate Pc-10) on eggs of *T. canis*. In order to do this, fertile nematode eggs were obtained by dissection of adult females fertilized specimens. After obtaining the eggs, they were inserted into 24 well plates previously filled with

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different concentrations of enzymatic extract of the fungus (100, 200, 400 and 500 µL). In addition, the behavior of *P. chlamydosporia* hyphae on *Toxocara canis* eggs was also observed in 2% water-agar medium (2% WA+ fungal isolate) when compared to the control group (2% WA + water). It was verified ovicidal activity with the enzymatic extract of *P. chlamydosporia* at concentrations of 400 and 500 **µ**L. At the same time, after 12 days of exposure of the *T. canis* eggs to *P. chlamydosporia* mycelia it was possible to observe the fungus action on eggshells, including penetration of the hyphae and colonization of the egg inside. The results confirm the ovicidal potential of the fungus and suggest its applicability in toxocariasis control programs.

Keywords: *Pochonia chlamydosporia*, *Toxocara canis,* Toxocariasis, Nematodes, Biological control.

1. INTRODUCTION

Helminthiases carried by the soil, which are known as geohelminthiases, are frequent and have their occurrence related to bad conditions of personal hygiene, health and environmental education (OLIVEIRA et al., 2011). Marques et al. (2012) and Zaia et al. (2015) argue that the presence of animal feces on soil in urban areas is a serious public health problem, as they may contaminate the environment with eggs, cysts, oocysts and larvae of parasites with anthropozoonotic potential. This problem is often associated with the presence of parasitized and non-worming stray dogs and cats in public and recreational places (PULLAN, 2012; MACPHERSON, 2013).

Toxocara canis and *Toxocara cati* are among the several species of helminths exhibited by samples of environmental matrices and disseminated by faeces of dogs and cats, denouncing that beach sand and public parks are important epidemic outbreaks of human infection, especially for children (KAPLAN et al., 2004; RUBINSKY-ELEFANT et al., 2010; SARAEI, 2012;). Ascarid eggs passed in the faeces of canids and felids in a suitable environment may remain infective for years and are capable of infecting not only canids and felids, but a large range of other

vertebrate paratenic hosts, including man. Infection with *Toxocara* species also occurs following the ingestion of paratenic hosts containing infective larvae (WU and BOWMAN, 2020).

The adult form of *Toxocara* spp. is established in the small intestine of dogs and cats infected by accidental ingestion of embryonated eggs or parasitic paratenic hosts (ALDAWEK et al., 2002). It is also worth mentioning that the infection may occur either by a transplacental route, verified only for *T. canis*, or by a transmammary route, characterized by ingestion of larvae (L3) together with colostrum or contaminated milk from dogs or cats infected (LESCANO, QUEIROZ and CHIEFFI, 2004; SANTOS, 2013). Eggs containing infective third-stage larvae are accidentally ingested by human beings through contact with contaminated food, water, soil, or utensils. In the small intestine, the larvae are released from the eggs, penetrate the intestinal wall, and travel via the circulatory system to various organs, including the lungs, liver, muscles, and central nervous system (PAWLOWSKI, 2001; MA, 2018).

According to Araújo et al. (2009), among the numerous measures related to helminth control, the use of nematophagous fungi as biological control agents stands out, enabling the substitution or reduction of residues generated by synthetic chemicals, responsible for causing negative impacts to the environment and health human and animal.

Authors have demonstrated the ability of *Pochonia chlamydosporia* to develop in the soil organic matter in the presence of nematodes, allowing the colonization and the unfeasibility of helminth eggs (STIRLING, 1991; MONTEIRO et al., 2017). In addition to the direct effect of fungus parasitism on embryonic development, there is the enzymatic action on the nematode eggshell, which increases its permeability and facilitates the passage of possible toxins produced by the fungus that also carry an embryotoxic action. Moreover, the ability of this fungus to produce many chlamydospores, not to be pathogenic to humans and other animals and to be easily produced in vitro, configures it as a very promising alternative environmental and sustainable method in the control

of several geohelminthiases.

Authors have used the fungus as a biological control at various stages of the *Toxocara* cycle and demonstrated its ovicidal potential (CARVALHO et al., 2010; ARAÚJO et al., 2012; MACIEL et al., 2012 and HIURA et al., 2015). Braga et al. (2011) working with enzymatic extract of *P. chlamydosporia* on eggs of *Ancylostoma* spp., registered a percentage of 76.8% in the reduction of hatchability of nematode larvae. Despite this study, the characterization of the ovicidal potential inherent to the *P. chlamydosporia* enzymatic extract on *T. canis* eggs at different dosages has not yet been elucidated. Therefore, the present study aimed to evaluate the embryotoxic action of different concentrations of *P.chlamydosporia* proteases on *T. canis* eggs under laboratory conditions. Additionally, the interaction between fungal mycelium and nematode eggs was characterized.

2. METHODS

Eggs retrieval

Toxocara canis eggs were obtained by dissection of the uterus of adult females from wandering, undetermined and parasitized dogs from the southeast region, Viçosa, latitude 20 ° 45'14 "S, longitude 42 ° 52'55" W, Minas Gerais, Brazil. The identification of adult specimens and eggs was conducted based on the descriptions of Anderson (1999). After obtaining the eggs, they were washed twice in 0.9% saline solution and subsequently sedimented in a glass Falcon tube (15 ml) for 20 minutes. Then, the eggs were morphologically analyzed for their integrity and viability by light microscopy, objective lens of 40 x, following the criteria described by Araújo et al. (2009). The eggs were then incubated at 30 ± 2° C for 4 days in saline solution to induce embryogenesis.

Enzymatic extraction of the fungus

Through electrophoretic analyzes conducted in the laboratory of Immunochemistry and Glycobiology, from the Federal University of Viçosa (UFV), Minas Gerais, Brazil, the enzymatic dosage of proteases obtained from the extract of *P. chlamydosporia* was carried out. For this, 1.5 mg of the commercial fungus formulation (Rizoflora®) was weighed into 15 ml sterile Falcon tube. Then, a solution of 10 ml of PBS (0.1

M and pH 7.2-7.4) (phosphate-saline buffer solution) at room temperature was poured promoting the resuspension of the fungal formulation. Subsequently, such solution was centrifuged at 3000 rpm for three minutes for collection and sterilization of the supernatant in the flow chamber.

After the extraction, the extract was inoculated in a 24-well plate at dosages of 00, 200, 400 and 500 µL and the control (H2O). The analyzes were carried out in triplicate using 15 partially embryonated eggs in each well / dose, totaling 45 eggs per dosage. The samples were then incubated in biochemical oxygen demand (B.O.D) at 35ºC in the dark and analyzed at 0, 24, and 72 hours and the analyzes took place through optical microscope views (100x objective).

In vitro fungal mycelium assay

The fungal isolate (Pc-10) used in this study was kept in a refrigerator at -4ºC in the Laboratory of Immunochemistry and Glycobiology of the Federal University of Viçosa, MG, Brazil. After its obtaining, the isolate was poured into petri dishes previously containing 10 ml of 2% agar-water for the establishment of the fungus. Subsequently, 50 eggs of *T. canis* were inoculated in medium containing *P. chlamydosporia* (treated group), as well as in medium without the fungus (control group). Then, the plates were incubated in B.O.D and maintained under 27 ± 2 ° C for 12 days and the analyzes took place through visualizations with the stereomicroscope and optical microscope (100x objective).

Statistical analysis

The statistical model used in the study was carried out in a completely randomized design. And data from the experimental test over the intervals were submitted to the Friedman test ($p < 0.05$) conducted in the statistical program Bioestat 5.4.

3. RESULTS AND DISCUSSION

After the incubation of *T. canis* eggs it was possible to demonstrate that the enzymatic extract of *P. chlamydosporia* promoted ovicidal effect in relation to the control group. However, in a study developed by Frassy et al. (2010), the

authors found that the ovicidal action of *P. chlamydosporia*, which grew in 2% WA medium, over *T. canis* eggs was 43.8% in relation to the control group.

These results agree with those previously published by Frassy et al. (2010) and affirm the use of *P. chlamydosporia* as an alternative for biological control of *T. canis*. There are a few studies involving *Pochonia fungal* enzyme extract in *Toxocara* eggs (BRAGA et al., 2010).

According to Braga et al. (2011) and Ward et al. (2012), the fungus *P. chlamydosporia* produces a series of proteases, such as VCP1, which is responsible for the destruction of nematode eggs. These authors also point out that this protease acts on the degradation of the protein layer that constitutes the outer membrane of the *T. canis* eggs, thus enabling its embryotoxic or ovicidal effect. In addition, the same authors evaluated the ovicidal action of the enzymatic extract of *P. chlamydosporia* on eggs of *Ancylostoma* sp. and verified impairment of viability in 76.8% of the eggs. Despite this study and to our knowledge, no research has yet characterized the ovicidal effect of *P. chlamydosporia* enzymatic extract on *T. canis* eggs. However, Lysek and Sterba (1991) state that the occurrence of chemical events characterized by enzymatic secretion by hyphae bodies is one of the main mechanisms observed during the stage of fungi penetration through eggs.

The lower ovicidal efficiency observed by the enzymatic extract can be partly justified by the serial dilutions obtained in the present study (Table 1), as well as the shorter exposure time with the nematode eggs.

Table 1. Relationship established between the total volume (µL) of the enzymatic extract of the fungus *Pochonia chlamydosporia* and enzymatic activity (IU) on *Toxocara canis* eggs. Data were expressed as mean ± standard deviation and percentage of affected eggs.

Dosage (µL)	Enzymatic activity (IU)	Eggs unfeasibility rate (%)
500	16,33 ± 3,21 a*	98
400	8,66 ± 1,52 ab	52
200	4,33 ± 1 b	26
100	2,00 ± 1,15 b	12
Control (H_2O)	0 ± 0	0

* Means followed by the same letters do not differ from each other p (<0.05).

T. canis egg is highly resistant (Figure 1), condition that is justified by the presence of the three layers that constitute the eggshell (vitelline layer, chitinous layer and lipid layer). Based on the results obtained in comparison with the control group (Figure 2A), it is possible to observe that the dosages of 100 and 200 µL (Figure 2B) did not induce significant structural modifications in the egg integument. The dosages of 400 and 500 µL (Figure 2C, D) resulted in a greater ovicidal effect, due to the rupture of the layers that make up the egg wall, leading to the inviability of 98% of exposed eggs. According to Lysek (1976), a fungus is considered ovicidal when it has the capacity to impair the embryogenesis process of helminth and, consequently, parasitic development. In the current study, it was possible to observe that the dosage of 500 µL presented a high significance in the disruption of the structures the eggshell (Figure 2D) favoring the death of the developing embryo. Moreover, the membrane rupture would be favored, once the proteases make the outer tegument of the eggs softer and thinner, facilitating rupture of the ovular surface (BOJANICH; BASUALDO and GIUSANO, 2017).

Figure 1. Non-embryonated eggs of *Toxocara canis*. Optical microscopy

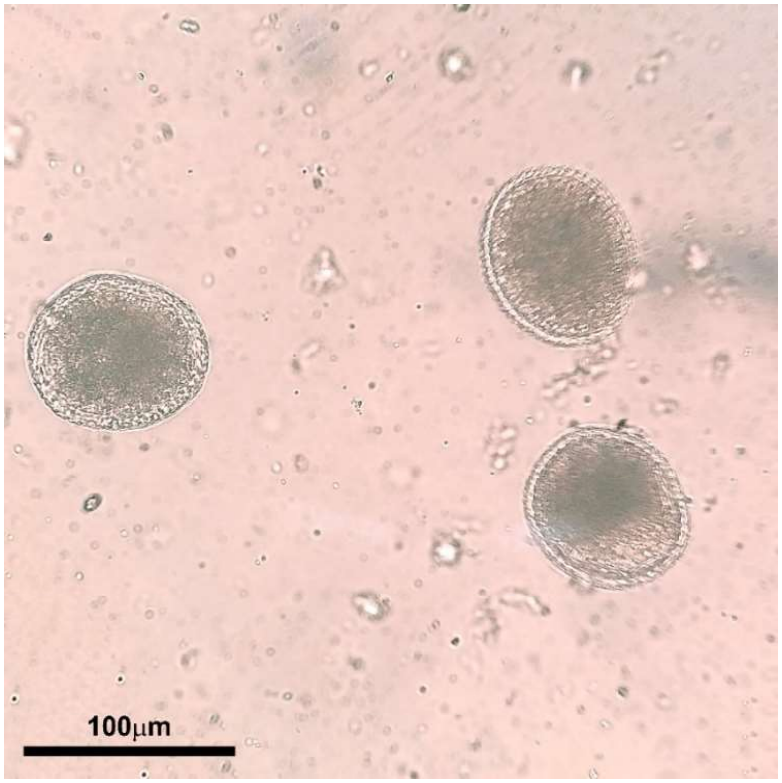
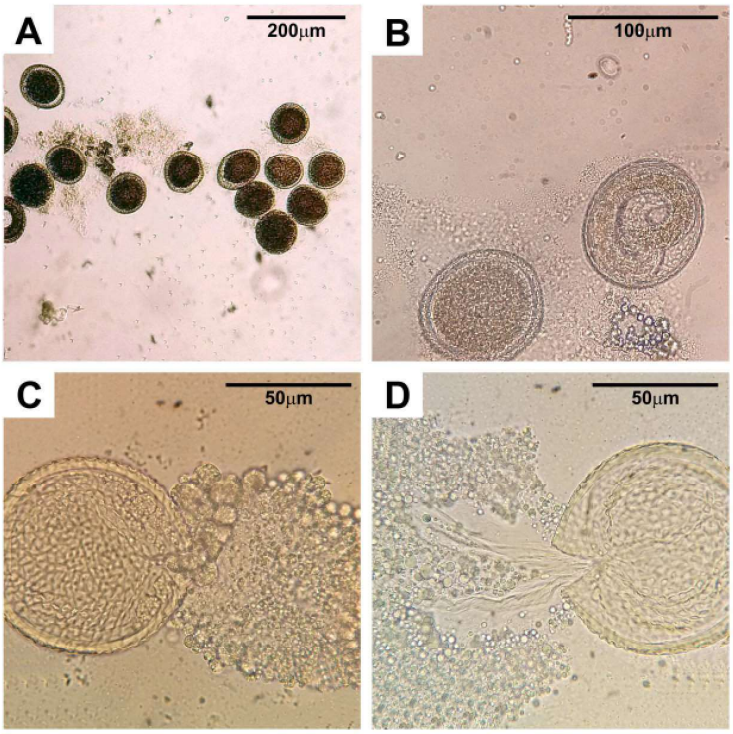


Figure 2. Activity of the enzymatic extract on the eggs of *Toxocara canis*. Optical microscope. A) Control group (H2O) of larval eggs. B) Eggs in dosages of 100 and 200 µL. C and D) dosages of 400 µL and 500 µL on eggs.



The results evaluated at the 12th day of incubation at 29º C revealed the effects type 1, 2 and 3. During the experiment, the hyphae established contact with the eggs of *T. canis* from the 5th day of exposure, impairing their integrity and, therefore, the embryo formation and proportional hatching of the larvae by 68% when compared to the control group (Table 2).

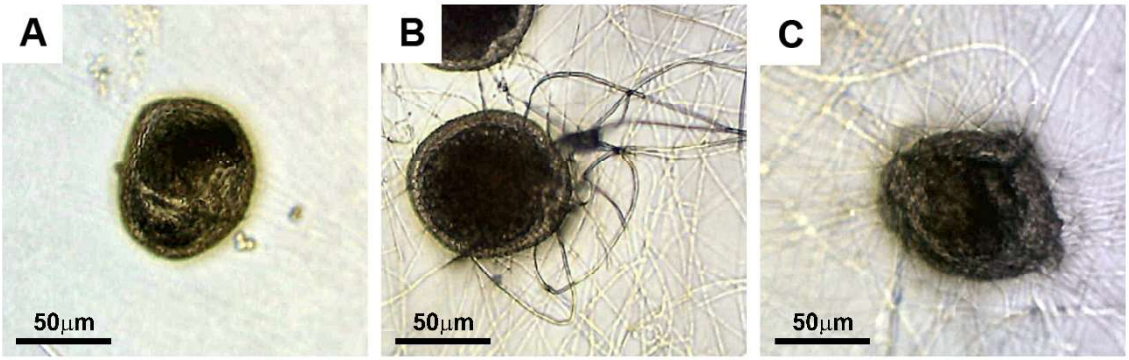
Table 2. Evaluation of the inviability of eggs of *Toxocara canis* and the fungus *Pochonia chlamydosporia* submitted to in vitro tests after 12 days. Data expressed as mean ± standard deviation.

Treatment	Effect type 1	Effect type 2	Effect type 3
Pc-10 + *Tc*	45,3 ± 4,72 a*	34,0 ± 10,53 ab	2,0 ± 1,0 c
Control (*Tc*)	0 ± 0	0 ± 0	0 ± 0

* Means followed by the same letters do not differ from each other p (<0.05).

In agreement to the results demonstrate by Frassy et al. (2010), studying the interaction between eggs of *T. canis* and *P. chlamydosporia*, it was possible to observed , by light microscopy, interactions of the fungus type 1 (Figure 3A). These interactions were characterized by a physiological and biochemical effect, without causing any morphological damage to the eggshell, where the hyphae are observed adhering to the integument. Interactions of the fungus type 2 (Figure 3B), in which is observed a lytic effect with morphological alterations of the integument and the embryo inside the egg, without penetration of hyphae through the tegument. And the interaction of type 3, which was formed by lytic effect with significant morphological changes of the embryo and the integument of the eggs, due to the penetration of hyphae and internal colonization of the egg (Figure 3C).

Figure 3. Behavior of the hyphae of the fungus *Pochonia chlamydosporia* on eggs of *Toxocara canis* in vitro observed after 12 days. Optical microscope. A) Adherence and contact of the hyphae in the vitelline membrane (external). B) Contact and penetration of hyphae. C)Lytic effect and alteration in egg membranes.



The biological control established by *P. chlamydosporia* on *T. canis* is favored by proximity of the nematode eggs to the mycelium of the fungus, inducing its germination (ESCUDERO et al. 2016). As soon as the fungus contacts the eggs of the nematode, intense conidiogenesis occurs, and the hyphae begin to nourish and ramify by gradually destroying, through the secretion of enzymes, the covering membranes of the egg and, therefore, the embryo in development. When there are no more nutrients, the microorganism leaves the egg, initiating the final process of its own destruction.

4. CONCLUSION

In the present study, it was possible to verify that the isolate of *P. chlamydosporia* (Pc-10) showed a significant ovicidal effect on *T. canis* eggs. The extraction of enzymes from ovicidal fungi showed significant interference with the viability and integrability of *T. canis* eggs. However, there is a need for more studies to better elucidate the good use of the enzymes and the dosages that favor a greater ovicidal effect on the eggs of the nematode in question.

CONFLICT OF INTERESTS

Other authors have nothing to declare.

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ATIVIDADE ENZIMÁTICA DO FUNGO *Pochonia chlamydosporia* (SORDARIOMYCETES: HYPOCREALES) e seu potencial ovicida em ovos de *Toxocara canis* (NEMATODA: ASCARIDIDA)

Resumo

O controle biológico é considerado uma das medidas alternativas mais empregadas no combate a helmintos de relevância em medicina veterinária e em saúde pública. Dentre tais parasitos, *Toxocara canis* se destaca por sua elevada prevalência e ampla distribuição mundial, além de ser o principal causador da *larva migrans* visceral no homem. O presente trabalho objetivou demonstrar a atividade ovicida do fungo *Pochonia chlamydosporia* (isolado Pc-10) sobre ovos de *T. canis*. Para isso, ovos férteis do nematoide foram obtidos mediante dissecação de exemplares fêmeas

adultas fecundadas. Após obtenção dos ovos, estes foram inseridos em placas de 24 poços previamente preenchidos com diferentes concentrações de extrato enzimático do fungo (100, 200, 400 e 500 **µ**L). Ademais, o comportamento das hifas de *P. chlamydosporia* sobre os ovos de *T. canis* foi também observado em meio ágar-água 2% (AA2% + isolado fúngico) quando comparado ao grupo controle (AA2% + água). Foi constatada atividade ovicida com o extrato enzimático de *P. chlamydosporia* em concentrações de 400 e 500 **µ**L. Paralelamente, após 12 dias de exposição dos ovos de *T. canis* aos micélios de *P. chlamydosporia,* foi possível observar ação do fungo sobre a casca dos ovos, inclusive com penetração das hifas e colonização do interior do ovo. Os resultados ratificam o potencial ovicida do fungo e sugere sua aplicabilidade em programas de controle da toxocaríase.

Palavras-chave: *Pochonia chlamydosporia*, *Toxocara canis,* Toxocaríase, Nematoides, Controle biológico.

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