Brazilian Journal of Veterinary Parasitology

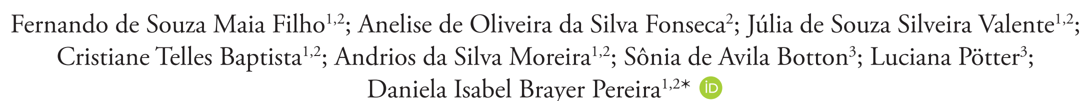
Original Article

ISSN 1984-2961 (Electronic)
www.cbpv.org.br/rbpv

Braz. J. Vet. Parasitol., Jaboticabal, v. 28, n. 1, p. 91-96, jan.-mar. 2019
Doi: https://doi.org/10.1590/S1984-29612019007

Exposure of *Toxocara canis* eggs to *Purpureocillium lilacinum* as a biocontrol strategy: an experimental model evaluation

Exposição de ovos de *Toxocara canis* a *Purpureocillium lilacinum* como estratégia de biocontrole: uma avaliação em modelo experimental



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Received September 17, 2018
Accepted December 12, 2018

Abstract

Purpureocillium lilacinum is a nematophagous fungus used in biological control against some parasites, including *Toxocara canis*. This study researched the infectivity of embryonated *T. canis* eggs after exposure to the fungus *P. lilacinum*. *T. canis* eggs were exposed to *P. lilacinum* for 15 or 30 days and subsequently administered to Swiss mice (n=20). Control group consisted of mice who received *T. canis* embryonated eggs without fungal exposure. Forty-eight hours after infection, heart, lung, and liver from animals of each group were collected to assess larval recovery. The organs of mice that received embryonated eggs exposed to the fungus showed a lower average larval recovery (P<0.05) suggesting that exposure of *T. canis* eggs to *P. lilacinum* was able to reduce experimental infection. Under the evaluated conditions, the interaction time between the fungus and the parasite eggs was not a significant factor in larvae recovery. *P. lilacinum* may be considered a promising *T. canis* biological control agent. However, further studies are needed to determine a protocol for the use of this fungus as a biological control agent.

Keywords: Geohelminths, ovicidal fungi, public health, environmental health, biological control.

Resumo

Purpureocillium lilacinum é um fungo nematófago com potencial para uso no controle biológico de parasitos, incluindo *Toxocara canis*. Este estudo pesquisou a infectividade de ovos de *T. canis* embrionados após exposição ao fungo *P. lilacinum*. Ovos de *T. canis* foram expostos ao fungo por 15 ou 30 dias e subsequentemente administrados a camundongos Swiss (n=20). O grupo controle consistiu de camundongos que receberam ovos embrionados do parasita sem exposição ao fungo. Quarenta e oito horas após a infecção, coração, pulmão e fígado dos camundongos foram coletados para avaliar a recuperação larval. Os órgãos dos animais que receberam ovos embrionados expostos ao fungo apresentaram menor média de recuperação larval (P<0,05) do que os infectados com ovos sem exposição ao fungo, sugerindo que a exposição dos ovos de *T. canis* a *P. lilacinum* foi capaz de reduzir a infecção experimental. Nas condições avaliadas, o tempo de interação entre o fungo e os ovos do parasito não foi um fator significativo na recuperação das larvas. *P. lilacinum* pode ser considerado um promissor agente de controle biológico de *T. canis*, no entanto, mais estudos são necessários para avaliar o emprego deste fungo como um agente de controle biológico.

Palavras-chave: Geohelmintos, fungos ovicidas, saúde pública, saúde ambiental, controle biológico.

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Introduction

Purpureocillium lilacinum, formerly known as *Paecilomyces lilacinus*, is a filamentous, soil saprobe fungus that is able to grow in wide pH ranges and uses different substrate sources (LUANGSA-ARD et al., 2011). This species of fungus has played a relevant role in the biological control of various parasites, such as *Toxocara canis* (BASUALDO et al., 2000; GORTARI et al., 2008; CARVALHO et al., 2010), *Taenia hydatigena* (CIARMELA et al., 2005), *Taenia saginata*, *Moniezia* sp. (BRAGA et al., 2008a,b), *Dipylidium caninum* (ARAUJO et al., 2009), *Oxyuris equi* (BRAGA et al., 2012), *Fasciola hepatica* (NAJAFI et al., 2017) and *Ancylostoma* (HOFSTÄTTER et al., 2017).

Nematophagous ovicidal fungi used in parasite biological control are able to colonize and digest nematode eggs in the soil. The egg infection begins with the growth of the fungal hyphae on the egg, which penetrate the egg wall via mechanical and/or enzymatic activity and rapidly colonize the entire egg (HUANG et al., 2004).

T. canis is an intestinal parasite of dogs, whose infection of paratenic hosts occurs mainly by accidental ingestion of embryonated eggs. Toxocariasis is a worldwide public health problem (CHEN et al., 2018; FAKHRI et al., 2018), because human exposure to this disease can be enhanced by environmental contamination, favored by the presence of dogs infected with *T. canis*, as well as the resistance of eggs in the soil. Places such as parks, squares, and beaches increase exposure of children to *T. canis* infection, especially those who are about five years of age, because of geophagia and nail biting habits (MACPHERSON, 2013). Humans behave as paratenic hosts of *T. canis*, preventing the full development of the helminth, but the larvae can survive for long periods in the body, migrating to different organs and tissues, where they remain encysted and viable resulting in diverse clinical manifestations, which are characterized as visceral larva *migrans*, ocular larva *migrans*, and covert toxocariasis syndromes (CHEN et al., 2018).

Given the problems caused by chemical control, especially its harmful effects on human health and the environment, the development of alternative methods of biological control is of paramount importance (MAIA et al., 2017). In this sense, biological control is a natural tool and an environmentally friendly alternative in the control of parasites of medical and veterinary relevance. This study investigated the experimental infectivity of embryonated *T. canis* eggs after exposure to the fungus *P. lilacinum.*

Materials and Methods

Fungal isolate

P. lilacinum used in this study was kindly provided by CENARGEN (National Research Center for Genetic Resources and Biotechnology, Brazil).

T. canis embryonated eggs

T. canis eggs were obtained by female parasite hysterectomy, as described by Maia et al. (2013). The eggs were put in a 2% formalin, 0.05% streptomycin sulfate, and 0.01% chloramphenicol solution. Embryonation was achieved by incubating the solution at 25°C for 30 days with daily aeration.

P. lilacinum interaction with T. canis eggs

Fungal culture discs were transferred into 10 Erlenmeyer flasks containing 150 ml modified liquid minimal medium [NH_4NO_3 (0.4 g/L); $MgSO_4 7H2O$ (0.12 g/L); $Na_2HPO_4 7H2O$ (3.18 g/L); KH_2PO_4 (0.26 g/L); yeast extract (0.3 g/L)]. The flasks were incubated in a bacteriological incubator at 25°C with daily manual stirring for 15 days. On the 15th day, 500 embryonating *T. canis* eggs were added to each flask containing the fungal culture and incubated under the same conditions described above for an additional 15 or 30 days. Additionally, 10 flasks containing parasite eggs (with no fungal contact) were maintained under the same culture conditions as the control group (15 or 30 days). At the end of the fungus and egg interaction periods, the culture medium was centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml 0.01 M phosphate buffer solution, pH 7.4 (PBS). To count the eggs and viability evaluation, 10 µL of the solution was analyzed by light microscopy at a magnification of 400X. The eggs were considered viable when there was larva inside.

Scanning Electron Microscopy (SEM)

To perform SEM, *P. lilacinum* culture discs were transferred into four Erlenmeyer flasks containing 100 mL modified liquid minimal medium incubated in a bacteriological incubator at 25°C with daily manual stirring for 15 days. On the 15th day, 500 embryonated *T. canis* eggs were added to flasks containing the fungal culture and incubated under the same conditions described above. Two flasks were incubated for 15 days and the other two flasks were incubated for 30 days. Two flasks containing parasite eggs (with no fungus contact) were maintained under the same culture conditions as the control group (15 and 30 days). By the end of the evaluation period (15 and 30 days) the samples were fixed in 3% glutaraldehyde and 0.05 M phosphate buffer (1000 µL glutaraldehyde: 300 µL culture), at pH 6.8, and maintained at 4°C for 48 hours. Subsequently, the cultures were fractionated into Falcon tubes and centrifuged for 15 minutes at 8000 rpm, discarding the supernatant. The precipitate was then subjected to successive ethanol baths (30, 50, 70, and 95%) and kept in 100% ethanol for 15 minutes. After this period, the samples were transferred to Petri dishes and dried in a bacteriological oven at 37°C for 3 hours. After drying, the samples were fixed to the stubs with double carbon tape and covered with gold in the sputtering equipment (Denton Vacuum Desk V) for 120 seconds at 19 mM, being visualized at 15 KV, under magnification from 400 to 2,500X (Jeol, JSM - 6610LV, EUA).

Experimental model

Eight-week-old female Swiss mice (*Mus musculus*) were acquired from the animal facility at Universidade Federal de Pelotas (UFPel). The animals were kept in proper cages room at

temperature of 25°C, receiving food and water *ad libitum*. In the first experiment, control group (n=20) animals were infected by oral route with with 0.2 mL PBS containing 100 embryonated *T. canis* eggs. The treatment group (n=20) consisted of mice that were infected with 0.2 mL PBS containing 100 embryonated *T. canis* eggs, which had been exposed to *P. lilacinum* for 15 days. The second experiment was carried out under the same conditions as the first, but the eggs that were given to the treatment group mice were exposed to the fungus for 30 days.

Forty-eight hours after infection, the mice from both experiments were euthanized through cervical dislocation as approved by the Ethics Committee on Animal Experimentation protocol of the UFPel (CEEA 0678). The liver, lung and heart of all animals were removed for larval recovery. The organs were macerated and digested in 50 ml 1% hydrochloric acid and 1% pepsin solution by constant stirring at 120 rpm at 37°C overnight. Subsequently, the digested organs were subjected to centrifugation at 2000 rpm/5 minutes. The supernatant was discarded and the total precipitate of each organ was examined between a slide and cover slip under optical microscopy at magnifications of 100X and 400X for larval count (XI & JIN, 1998).

Statistical analysis

The data for larval counting from the digested organs in both experiments were subjected to a normality test using Kolmogorov-Smirnov. As the response variable did not show normality, data were submitted to the non-parametric Kruskal-Wallis test. Thereafter, the Mood's median test was applied to compare means two to two. The analysis was performed using SAS statistical software (version 9.4), with a 5% significance level.

Results

Microscopic analysis of embryonated eggs of the control group (*T. canis* eggs not exposed to the fungus) showed that the eggs were embryonated and intact (Figure 1A). However, the eggs that were incubated with the nematophagous fungus *P. lilacinum* were embryonated and colonized with fungal hyphae across their surface (Figure 1B). SEM observation revealed that the surfaces of the *T. canis* eggs in the control group were intact, and no destroyed eggs were observed (Figure 2A). However, in *T. canis* eggs exposed to *P. lilacinum* hyphae were observed colonizing the surface and the inside of the eggs, and subsequently destroying them. This effect was observed in eggs exposed to the fungus for 15 and 30 days (Figure 2B, 2C, and 2D).

Table 1 shows the average of *T. canis* larval recovery from heart, lung, and liver from mice experimentally infected with *T. canis* eggs exposed or not to *P. lilacinum*. The organs of the animals that received the embryonated eggs exposed to the fungus showed lower average recovery rate ($P<0.05$) than those from organs infected with embryonated *T. canis* eggs which were not exposed to the fungus (Table 1). Furthermore, there were no differences ($P>0.05$) between treatments when the periods of exposure of the eggs to the fungus were compared (Table 1).

Discussion

P. lilacinum, along with other nematophagous fungi, is a promising biocontrol agent because of its ability to capture and infect nematodes. *In vitro* studies evaluating the use of ovicidal fungi in *T. canis* biocontrol suggest that the use of these agents is an ecological and viable tool, complementary to other available

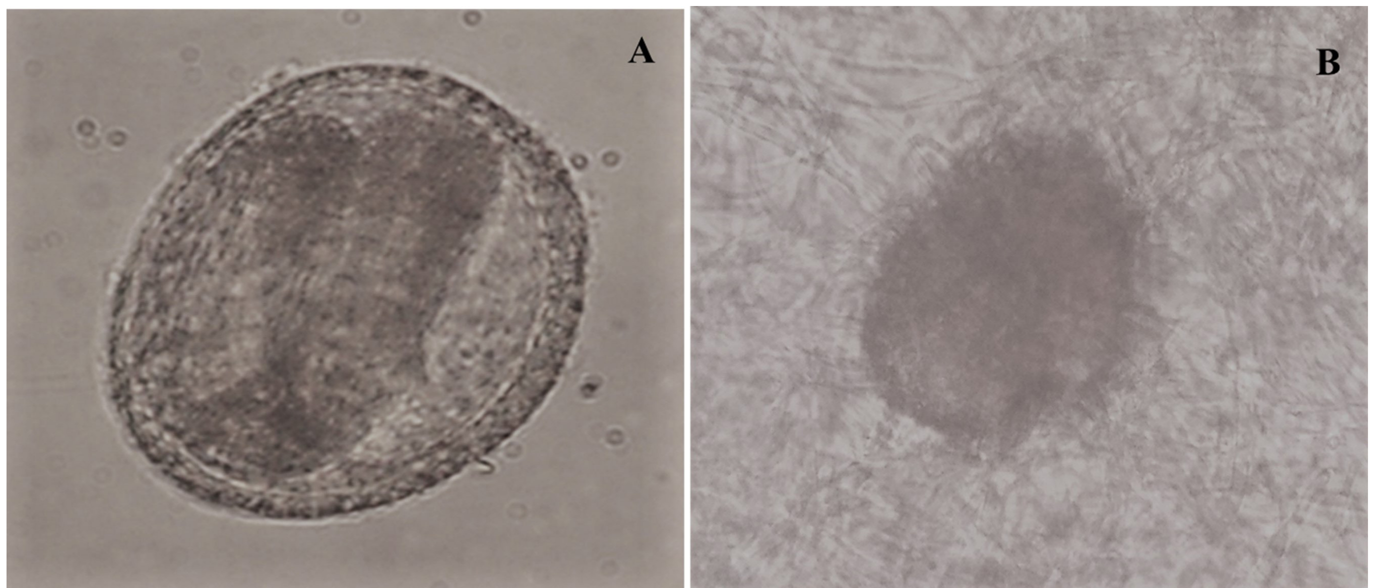

Figure 1. Light microscopy of *T. canis* eggs. (A) Control group: Embryonated *T. canis* egg not exposed to *P. lilacinum.* The eggs were incubated in minimal medium at 25°C for 15 and 30 days. No morphological alterations were observed (magnification 400X); (B) *T. canis* egg colonized by *P. lilacinum* hyphae after culturing in minimal medium at 25ºC for 15 and 30 days. Note the colonized egg with fungal hyphae across its surface. This effect was observed over both periods of incubation time.

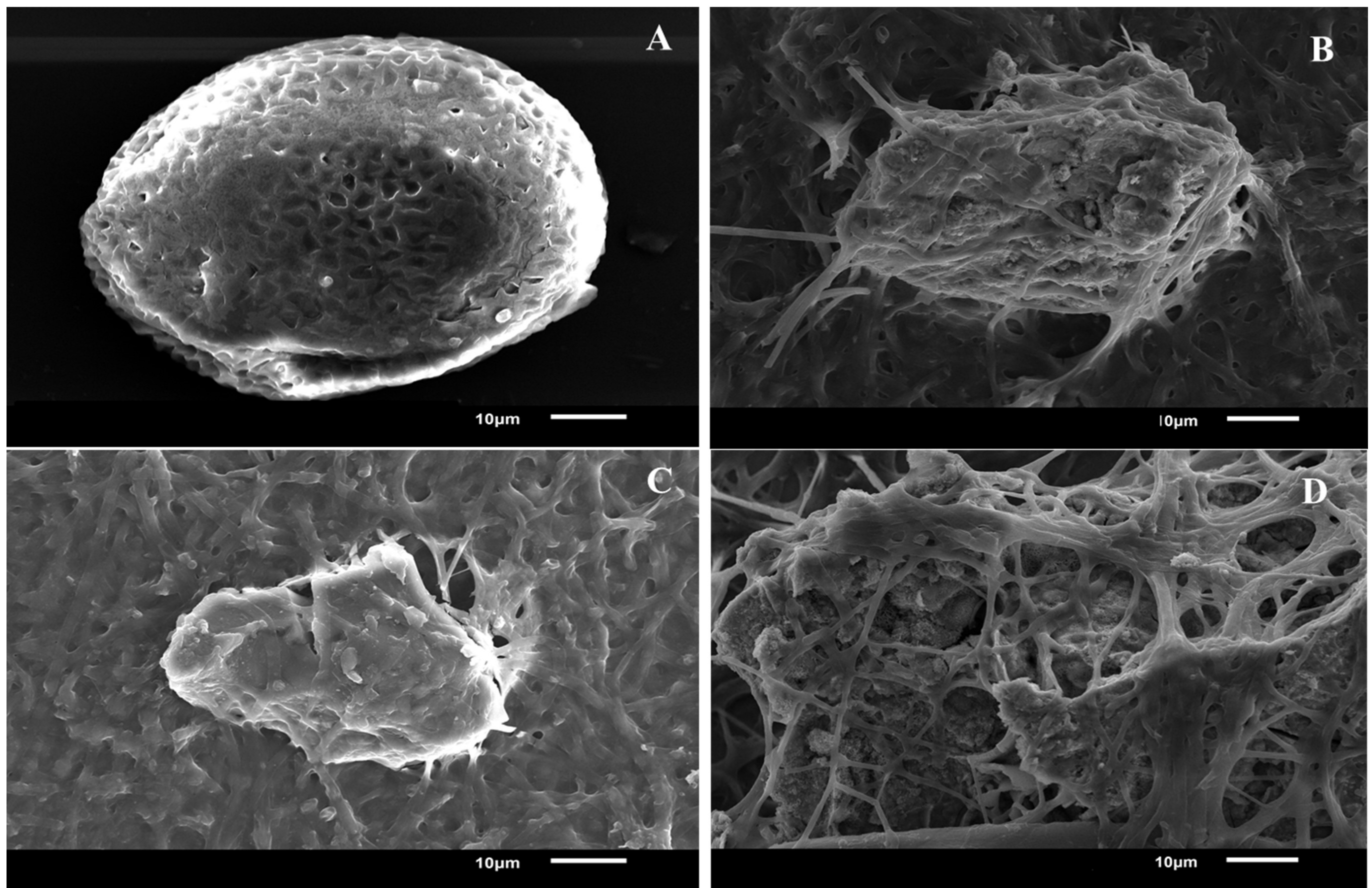

Figure 2. Scanning electron microscopy of *T. canis* eggs. (A) Control group: *T. canis* egg not exposed to *P. lilacinum. T. canis* eggs evaluated at 15 and 30 days, with surface intact and no destroyed eggs; (B) *T. canis* eggs exposed to *P. lilacinum* for 15 days and (C) 30 days. Hyphae colonizing the surface and interior of the egg are apparent for both periods of time; (D) *T. canis* eggs destroyed by *P. lilacinum.* Magnification 1400X.

Table 1. Mean count of *Toxocara canis* larvae in the organs of *Mus musculus* infected with *T. canis* eggs exposed to *Purpureocillium lilacinum.*

Mean count of *T. canis* larvae			
Organ	Control Group[1]*	Treatment (days)[2]	
		15	30
Liver	37.9[a]	19.7[b]	19.2[b]
Lung	14.6[a]	9.9[b]	9.5[b]
Heart	14.1[a]	9.5[b]	4.5[b]
Experimental group mean	66.6[a]	39.1[b]	33.2[b]

Different letters in the lines mean significant difference (P<0.05); [1]Animals infected with embryonated *T. canis* eggs; [2]Animals infected with embryonated *T. canis* eggs exposed to *P. lilacinum* for 15 or 30 days; *The values correspond to the mean of the control group for 15 or 30 days.

control measures used against gastrointestinal parasites of relevance to public health (BASUALDO et al., 2000; ARAUJO et al., 2009; CARVALHO et al., 2010; MAIA et al., 2013).

Our study used mice as experimental model to show the recovery of larvae from the tissues of animals infected with *T. canis* eggs previously exposed to *P. lilacinum* and observed that it was lower (P <0.05) than the ones from the organs of mice infected with embryonated eggs that had not been exposed to the fungus. The results of this study may be explained by the likely reduction in egg viability resulting from structural injuries caused by the fungi, and the damage to larval development, as already seen by Lysek (1978). In the present study, SEM analysis provided evidence of hyphae colonizing the surface and interior of *T. canis* eggs exposed to *P. lilacinum*, with subsequent damage and destruction of the eggs' layers. Similar effects on *T. canis* eggs were described by Basualdo et al. (2000), Ciarmela et al. (2002) and Araujo et al. (2012a) testing other nematophagous fungi.

Interestingly, our study also observed that the interaction time between *P. lilacinum* and *T. canis* eggs did not influence the fungus action on the eggs. There were no significant differences (P> 0.05) between the recovery of larvae at 15 and 30 days of exposure to the fungus (Table 1). Araújo et al. (1995) reported that the ovicidal effect (type 3 effect) of the fungus could occur from the seventh day of interaction. Maia et al. (2013), while evaluating the ovicidal effect of different kinds of isolated soil fungi on *T. canis* eggs, observed that type 3 effect occurring from the 14th day of egg-fungal exposure.

The ovicidal effect of the fungus on parasite eggs occurs by mechanical and enzymatic action. Although the pathogenic

mechanisms of nematophagous fungi are not yet fully understood, evidence shows that extracellular hydrolytic enzymes, including proteases, collagenases, and chitinases, may be involved in the penetration and digestion of nematode egg structures (HUANG et al., 2004; YANG et al., 2007). Nevertheless, several mechanisms of action have been suggested to explain the biological activity of *P. lilacinum* against nematodes. The main mechanism of action is by direct infection of the nematode eggs. *P. lilacinus* produces serine protease, which plays an important role in the penetration of the fungus in nematode eggs (BONANTS et al., 1995). In this research, we did not evaluate enzymatic effects of *P. lilacinum* on *T. canis* eggs. However, we did observe morphological alteration and destruction of *T. canis* eggs caused by exposure to *P. lilacinum*, as previously described by Basualdo et al. (2000).

In vivo studies reporting the use of nematophagous fungi are in their infancy. The literature includes few reports of predator nematophagous fungi of the genera *Duddingtonia*, *Monacrosporium*, and *Arthrobotrys*, where experimental passage of the fungus through the gastrointestinal tract of domestic animals is evaluated (ARAUJO et al., 2012b; TAVELA et al., 2013). Nevertheless, the *in vivo* exposition of helminth eggs with ovicidal nematophagous fungi and their subsequent effect on the infection process has been reported by Araujo et al. (2012a) and Maia et al. (2017).

This is the first report demonstrating the *in vivo* effectiveness of the nematophagous fungus *P. lilacinum* to reduce *T. canis* infection in experimental animals. Previously, our research group demonstrated that exposure of *T. canis* eggs to another nematophagous fungi (*T. virens*) for 15 days significantly reduced the number of larvae recovered from the organs of experimentally infected mice (MAIA et al., 2017).

The idea of using *P. lilacinum* as a biological control agent in contexts with high environmental *T. canis* egg contamination is promising. One of the benefits to public health would be a reduction in the risk of infection of susceptible individuals, especially children and the definitive host itself. Another advantage of this kind of biological control would be the contribution to environmental health as it could enable less use of chemicals, thus minimizing both the contamination of the environment with hazardous compounds and the selective pressure on parasites to evolve resistance to anti-parasitic drugs.

Conclusion

The exposure of *T. canis* eggs to *P. lilacinum* was able to reduce mice experimental infection. In addition, parasite-fungus interaction time beyond 15 days did not affect larval recovery. Thus, *P. lilacinum* may be considered a promising *T. canis* biological control agent. Nevertheless, further studies are necessary to evaluate the use of this fungus in the environment as a biological control agent.

Acknowledgements

The authors would like to thank the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) of Brazil for student and researcher scholarships.

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