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Predatory Activity of the Fungus *Pleurotus eryngii* on *Ancylostoma caninum* Infective Larvae

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Abstract

The objective of this study was to evaluate *in vitro* predatory activity of *Pleurotus eryngii* fungal isolates on *A. caninum* of dogs. In the treated group, 1000 *A. caninum* larvae and 50 microliters of the fungal isolatewere placed in each petri dish, and in the control group only 1000 larvae were placed. The *P. eryngii* fungal isolatereduced the average number of *A. caninum larvae* compared to control *($p < 0.01$)* and presented a reduction percentage of 47.56%. The average number of L3 recovered in the treated group was 32.2 (± 15.3) while the average in the control group was 72.88 (±27.0). Consecutive reduction was observed from day 1 to day 7 in relation to the number of larvae in both groups *($p < 0.01$)*. It is concluded that the fungus *P. eryngii* showed predatory activity on *A. caninum* L3, and may be a control alternative.

Keywords: Ancylostomids; Biological control; Nematophagous fungi

Introduction

Dogs, despite the benefits which present as pets, may be considered as reservoirs of infectious agents such as parasites, bacteria, viruses and fungi and may be a potential source of contamination and risk to human health. Due to its zoonotic potential, among the gastrointestinal helminths, *Ancylostoma spp* has attracted great attention. *Ancylostoma caninum* and *A. braziliense* are intestinal geohelminths from dogs and cats and have required great attention because of their zoonotic potential by means of direct soil contamination with the faeces of infected animals, since their infective larvae (L3) are present in this contaminated environment [1,2]. Nowadays alternative measures to control these and other domestic animal endoparasitosis are searched in order to decrease use of chemotherapeutics and, consequently, the reduction of pollutant levels in the environment and in animal originating products [3]. In order to improve this control, use of biological control is suggested as a viable and promising alternative that reduces infections caused by gastrointestinal helminth parasites present in the environment, and whose action takes place by means of living organisms that act as natural antagonists in the environment [1,4]. Among these antagonists are the nematophagous fungi. These fungi have predatory capacity on helminths and constitute an option for the control of free-living stages of helminths of dogs and cats [1,4]. Nematophagous fungi include those fungi which attack larvae and/or eggs from these animals and use them as a nutrient source [3,5].

The species of nematophagous fungi prey the L3 of gastrointestinal nematodes and have been studied for their potential as biological control agents of gastrointestinal nematodes from domestic animals [6,7]. In this sense, the *Pleurotus* genre produces Nematotoxin, a toxin capable of immobilizing the nematodes that come in contact with it [8]. This fungus may be found in decaying wood and also commercially cultivated in several countries for human consumption. Regarding the general mechanism of "attack" of fungi on L3 or even to helminth eggs, literature mentions that nematophagous fungi attack by various processes, including: (a) capture; (b) parasitism and (c) production of toxins and enzymes [9]. In relation to the extracellular enzymes, including serine proteases (EC 3.4.21), chitinases (EC 3.2.1.14) and collagenases (EC 3.4.24.3), these are important virulence factors that can degrade the main chemical constituents of the nematodes' cuticle and eggshell. According to Kwok et al. [10] aerial hyphae of *Pleurotus spp*. have produced small droplets of toxin on agar culture, which were identified as trans-2-decenedioic acid. Overall, the nematodes that come in contact with this toxin are paralyzed and subsequently invaded by hyphae. This attack mechanism on the nematodes was observed in several species of *Pleurotus spp*, such as *Pleurotus dryinus*, *P. euosmus*, and *P. eryngii*, among others. In another context, literature shows research regarding *in vitro* biological control of *A. caninum* L3. However, this is the first report of predatory activity of *P. eryngii* on this nematode.

Thus, the present study aimed to evaluate the efficiency of the fungus *Pleorotus eryngii* on *in vitro* control of *Ancylostoma caninum* L3.

Materials and methods

Obtaining of the fungal isolate

Nematophagous fungus *P. eryngii* was used. This isolate is derived from the Parasitology Laboratory of the Federal University of Viçosa, Minas Gerais, and Brazil. This isolate has been maintained by continuous transfer to culture medium containing 2% water-agar.

Obtaining conidia

Culture dishes with 4 mm diameter were extracted from the fungal isolates maintained in test tubes containing 2% corn-meal-agar (2% CMA) and transferred to Petri dishes of 9.0 cm in diameter containing 20 ml of 2% potato dextrose agar, kept at 25°C in the dark for 10 days. After the growth of the isolates, new 4 mm diameter culture dishes were transferred to Petri dishes of 9.0 cm in diameter containing 20 ml of 2% water-agar (2% WA) in which were added 1 ml of distilled water containing 1,000 *Panagrellus spp.* larvae, a free-living nematode, daily over a period of 21 days to induce formation of fungal conidia. When complete fungal growth was observed, 5 ml of distilled water was added to each Petri dish, and the conidia and mycelial fragments were removed using technique described by Araujo et al. [11].

Obtaining *Ancylostoma caninum* larvae

Fresh faeces were obtained from dogs living in the state of Espírito Santo. From these fecal samples about 3-5g of feces were taken for performance of the Willis-Mollay technique (fluctuation) in order to identify eggs present in the samples. After identification of *Ancylostoma caninum* eggs, fecal cultures were prepared with about 20 g of feces and they were incubated in a BOD incubation chamber fora period of 7 days. After this period the larvae were extracted and identified by the Baermann technique.

Experimental assay

An *in vitro* experimental assay was carried out. In the experiment the nematicidal activity of the fungus *P. eryngii* on *A. caninum* L3 and control group (no fungus) was evaluated. Thirty Petri dishes of 9.0 cm in diameter were prepared containing 20 ml of 1% WA, fifteen plates from the treated group and fifteen plates from the control group. In the treated group, in each Petri plate 30 µl of the solution containing the L3 (1000 *A. caninum larvae)* and 50 µl of fungal isolate (1000 conidia) were added. In the control group (without fungi) only the L3 were placed on the plates. For seven days, every 24 hours, 10 random fields of 4 mm in diameter in each plate from the treated and control groups were observed under a light microscope at 10x objective, and the number of larvae was counted in each of the fields. At the end of seven days, the non-predated larvae were recovered from the content of the petri dishes using the Baermann apparatus with water at 42°C [12].

Statistical analysis

The average of *A. caninum* L3 recovered was calculated. Data was interpreted by analysis of variance at significance levels of 1 and 5% of probability. The efficiency of larvae predation in relation to control was evaluated by the Tukey test at 1% probability. Subsequently, the average reduction percentage of L3 was calculated according to the following formula:

$$\% \text{ Reduction} = \frac{\left(\begin{array}{l}\text{Average of recovered } L_3 \text{ from control} - \\ \text{Average of recovered } L_3 \text{from treatment}\end{array}\right)}{\text{Average of recovered } L_3 \text{ from control}} \times 100$$

Results and Discussion

Biological control using nematophagous fungi has the potential to become an important strategy to control gastrointestinal helminths in domestic animals. Larsen e Nansen [13], have shown that the fungus *Pleurotus spp* has nematicidal activity and can therefore be considered a nematophagous fungus. Although there are studies that demonstrate the action of the fungus *Pleurotus spp* as a predator of nematodes larvae, there are no studies demonstrating its action on larvae of the Ancylostoma *spp* genre.

Here, the predatory activity of the fungus *P. eryngii* on *A. caninum* larvae was demonstrated, verifying that the fungal isolate was able to interact and prey on the larvae during the experiment. The average number of L3 recovered from the control group was significantly higher than the average from the treated group. The *P. eryngii* fungal isolate reduced the average number of *A. caninum* L3 compared to control *($p < 0.01$)* and presented a reduction percentage of 47.56%.The average number of L3 recovered in the treated group was 32.2 (± 15.3) while the average in the control group was 72.88 (± 27.0).

Thorn and Barron [8] were the first to report that wood decomposing fungi have the ability to capture, kill and digest nematodes. They showed that 11 species of fungi belonging to the order Agaricales, including *Pleurotus ostreatus*, have the ability to kill root-knot nematodes. Palizi et al. [14] reported that this species reduced the formation of cysts from the nematode *Heterodera schachtii* and Marino and Silva [15], deduced that the use of *Pleurotus ostreatus* reduces the number of root-knot nematodes and egg mass of *Meloidogyne incognita*.

Kwok et al. [10] cited that *P. ostreatus* and related species have specialized cells in hyphae capable of producing droplets of a substance containing toxins. The nematode when in contact with this substance suffers paralysis and lysis of the cuticle. Although alive, the nematode remains immobile and liquids outside theirtissues stimulate the growth of the fungus hyphae in its direction, in a process of chemotaxis. These hyphae penetrate, and when in the nematodes tissues digest them and absorb the released nutrients. Satou et al. [16] observed that *P. ostreatus* produce bubbles with anti-nematode activity. These bubbles promoted reduction of the nematodes "head" due to the release of linoleic acid in the solution. In the present study it was observed that the L3 of *A. caninum* suffered action of the fungal isolate *P. eryngii*, since it was possible to visualize the presence of hyphae and small droplets/bubbles inside and at the periphery of the larvae and due to the increased number of such droplets within the larvae over the 7 days (Figure 1). In trial conducted

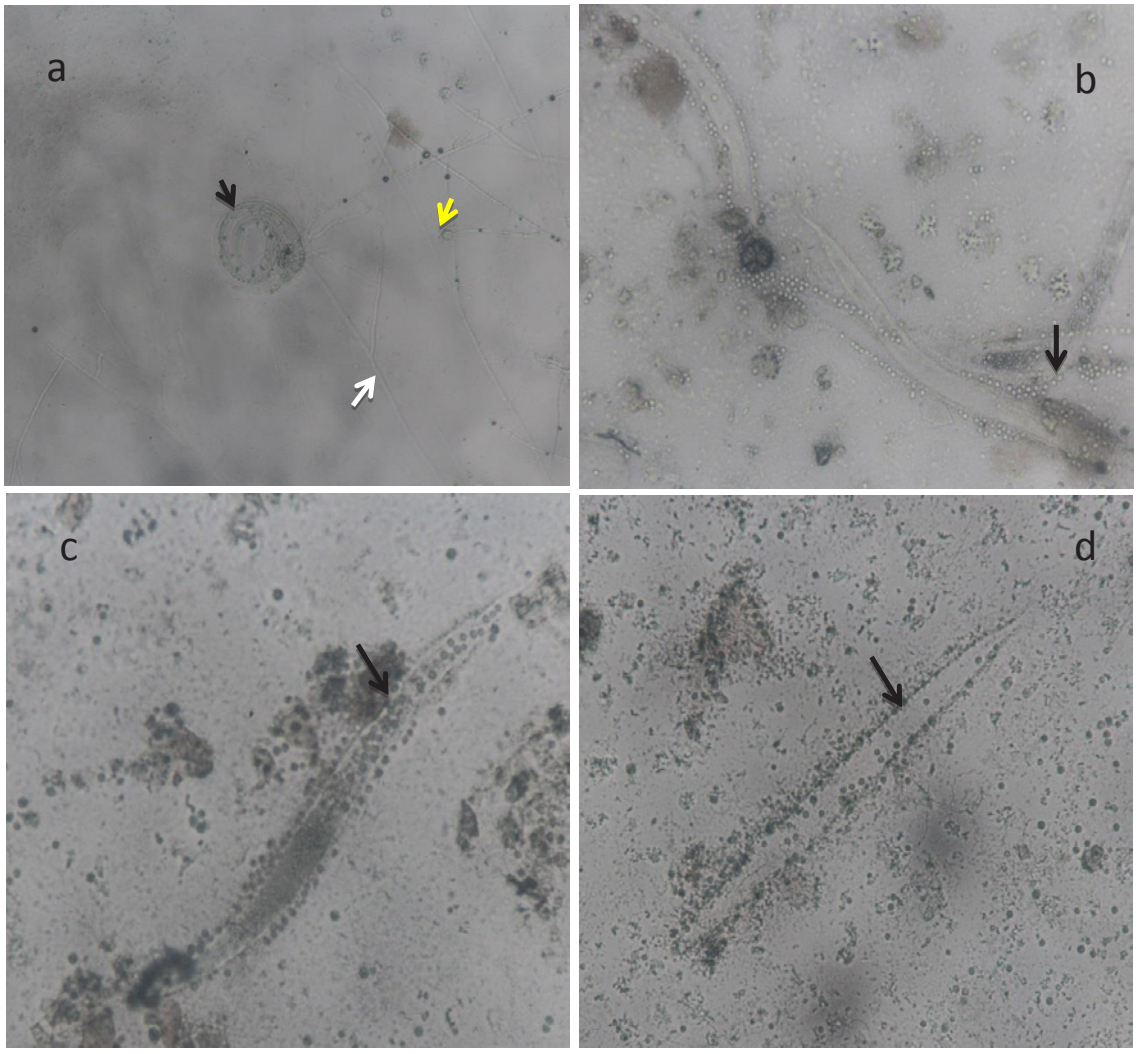

Figure 1: A) Larvae of *Ancylostoma caninum* (black arrow), hyphae of *Pleurotus eryngii* (white arrow) and droplet produced by the fungus (yellow arrow). B) Larvae of *Ancylostoma caninum* with droplets produced by the fungus *Pleurotus eryngii* in contact with the surface of the larvae (black arrow). C) Larvae of *Ancylostoma caninum* with droplets produced by *Pleurotus eryngii* inside the larvae (black arrow).D) Larvae of *Ancylostoma caninum* digested by the fungus *Pleurotus eryngii*. Light microscopy - 10x objective.

by Satou et al. [16] in which fungal isolates of *P. ostreatus* were used on free-living nematodes (Diplogastridae) spherical buttons were also observedby scanning electron microscopy and optical microscopy.

In the study involving the predatory activities of *Ancylostoma spp* L3, the works of Maciel et al. [17] demonstrated that nematophagous fungi from the *Arthrobotrys*, *Duddingtonia* and *Monacrosporium* genera presented *in vitro* predatory activity and at the end of the experiment observed percentage values of 88.76%, 97.75% and 89.89%, respectively. However, as mentioned earlier, this is the first report of *P. eryngii* activity on *Ancylostoma* L3 and comparatively it can be noted that, the studied fungus can be used in future experiments involving predation of potentially zoonotic helminth larvae, opening a new possibility of *in vitro* and *in vivo* studies.

The average of the larvae in the control and treated groups for 7 consecutive days of the experiment are shown in Table 1. Significant values were found *($p < 0.01$)* between the averages of the treated and control group, in which there was a consecutive decrease from day 1 to day 7 of the number of larvae in both treated and control groups. Significant difference *($p < 0.01$)* was also found between the days in the treated group compared to day 1 of treatment. These data corroborate with data found by other authors who also used fungal isolates of *Pleurotus spp*, but larvae of other nematodes.

Satou et al. [16] in microscopic examination of the inoculated fungus *(Pleurotus pulmonarius)* revealed the presence of secretory cells with tiny droplets of fluid across the agar surface and it became obvious that the larvae of parasites were immobilized by contact with the fungus within one to two hours, and no later than four hours of exposure there was a dramatic reduction in the number of mobile larvae of all species (*Ostertagia ostertagi,*

Table 1: Efficiency of *Pleurotus eryngii* fungal isolate in reducing the number of *Ancylostoma caninum* L3 larvae for 7 consecutive days.

	Average of the larvae (± SD)	
	Treated	**Control**
Day 1	7.6 (± 3.1) *	13.5 (± 5.1)
Day 2	6.6 (± 2.8) *	8.4 (± 4.8)
Day 3	6.4 (± 2.9) *#	5.0 (± 2.7)
Day 4	6.1 (± 2.1) *#	2.9 (± 2.8)
Day 5	4.9 (± 2.3) *#	1.8 (± 2.8)
Day 6	4.9 (± 2.4) *#	1.8 (± 2.9)
Day 7	5.0 (± 2.4) *#	1.5 (± 2.9)

* Comparison between columns: Average from the treated and control groups of the respective day differ from each other *($p < 0.01$).* # Comparison between lines: the average from the treated group differs *($p < 0.01$)* from the average from the treated group on day 1.

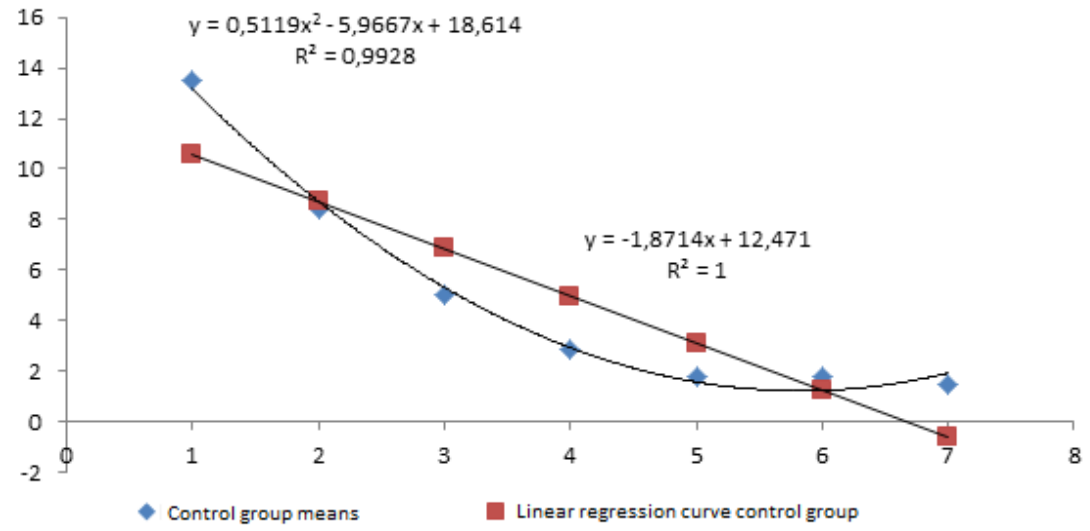

Figure 2: Linear regression curve calculated through the average of the *Ancylostoma caninum* larvae from the control group in function of time (days 1 to 7).

Cooperia oncophora, Oesophagostomum quadrispinulatum and *Cyathostomum)* and after eight hours, no more larvae motility was recorded.

In an experiment conducted by Graminha et al. [18] the pathogenicity of the fungus *Arthrobotrys musiformis* and *A. conoides* on infective larvae of *Haemonchus contortus* and *Ancylostoma spp* and embryonated eggs of *Ascaridiagalli* was evaluated and it was found that predation of both nematodes by *A. conoides* was crescent, obtaining the maximum predation on *Ancylostoma spp*. on the fourth day, whereas maximum *H. contortus* predation was only obtained on the sixth day and although the efficiency of both fungi was higher for *H. contortus* larvae than the ones from *Ancylostoma spp*., it was observed that predation tendency for both nematodes was crescent during the trial period.

The data presented in the present study showed that the number of L3 found in the control group decreased gradually throughout the experiment (Table 1). This can be explained by migration of L3 to the periphery of the petri dishes, since they migrate in search of a place in the medium with more nutrition and moisture. In a study by Larsen and Nansen [13], L3 of different nematodes (*Ostertagia ostertagi, Cooperia oncophora, Oesophagostomum quadrispinulatum* and *Cyathostomum* species*)* tended to distort the mycelium of the fungus *P. pulmonarius* by vigorous movement and after three or four hours the larvae were gathered around the periphery of the petri dishes where they were difficult to identify and count as movable or immovable, with data being recorded along the first three or four hours of exposure. This larval migration to the periphery of the plate where there is more moisture was also observed by Carvalho et al. [6] e Braga e Araujo [19].

Based on the values found in Table 1, the adjusted regression equations for the treated and control groups were estimated. The negative coefficients of linear regression indicates a descendent

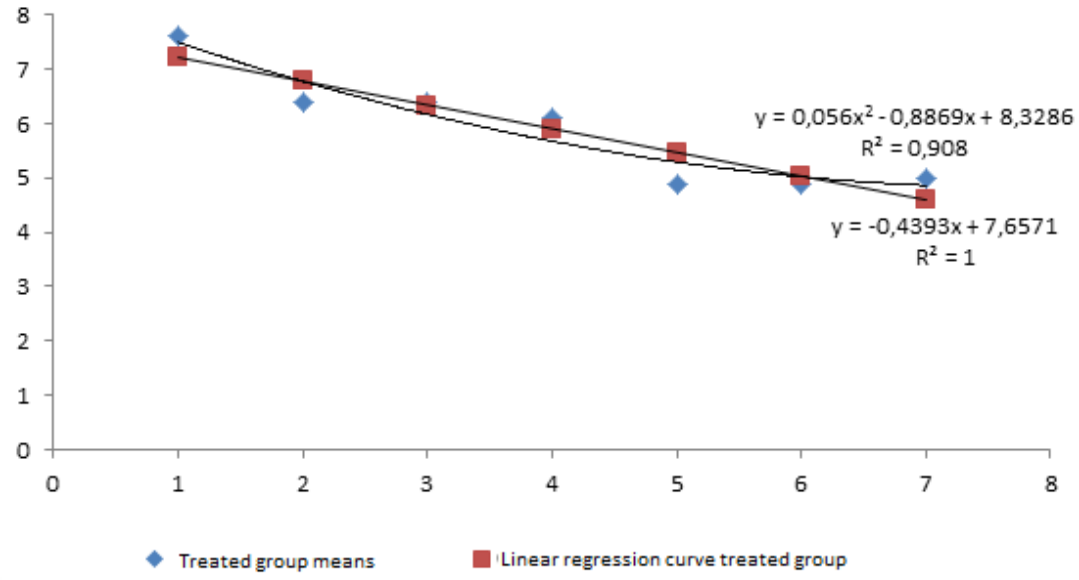

Figure 3: Linear regression curve calculated through the average of the *Ancylostomsa caninum* larvae from the treated group in function of time (days 1 to 7).

behavior of the regression curves, showing that the days directly influenced on predation of *A. caninum* L3, ie for each day added to the experiment, the average of the larvae decreased from 1.87 in the control (Figure 2) and 0.43 for treated group (Figure 3). The reduction of L3 in the control group during the study however was caused by the migration of the larvae to the periphery of the Petri dishes, place where the moisture level was higher.

Compared with other trials conducted with the same nematode (*Ancylostoma spp*), but with other nematophagous isolates (*Monacrosporiumthaumasium, Arthrobotrysrobusta*and *Duddingtonia flagrans*) the linear reduction coefficient was 0.23; 0.24 and 0.28 respectively in the different treated groups [17]. However, in a study by Braga et al. [20] predation efficiency of *Duddingtonia flagrans*, *Monacrosporium thaumasium*, *M. sinense* and *Arthrobotrys robusta* fungi on *Ancylostoma ceylanicum* L3 was established and the authors reported linear coefficient values in the treated groups of 0.73; 0.76; 0.78 and 0.82 respectively. Comparing the linear coefficient values of reduction found in this study with those recorded in the assays performed by Maciel et al. [17] it was observed that the fungus *P. eryngii* showed higher linear coefficient value (0.43), suggesting that for each day added to the experiment the average of L3 decreased, demonstrating a greater time influence on *A. caninum* larvae predationfrom the current study. However, when comparing the linear coefficient of reduction of the present study with Braga et al. [20] the linear coefficient reduction values were higher for all fungal species studied.

The results obtained in this study confirm previous reports of nematophagous fungi efficiency in the control of potentially zoonotic nematode larvae. It is estimated that about 1 billion people are currently infected with geohelminths, mainly due to contact with the soil, indicating that this is an important route of human infection, which is associated with serious health consequences if untreated [19]. Furthermore, studies on parasites that infect domestic animals have caused great interest due to the close relationship between man and animals, which may be a public health problem. Among the helminths with zoonotic potential, one can highlight those of the *Ancylostoma* genre [5].

Therefore, it is necessary to know the role of all organisms involved in the biocontrol of nematodes [19]. According to Araújo et al. [5] it is essential that funding agencies support research in this area because many basic aspects of biology, epidemiology and host-pathogen interaction need to be studied. To the industry it would fit the very important role of developing commercially viable formulations. Helminths are the conduits of soil-transmitted infections and a major health problem. In this study, the nematicidal activity of *Pleurotus eryngii* on the *Ancylostoma caninum* L3 was confirmed by the reduction in the number of larvae after treatment with the fungus.

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