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Fungal ovicidal activity on *Toxocara canis* eggs

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ABSTRACT

Background: Visceral toxocariasis is a parasitic zoonosis caused by *Toxocara canis*. The prevalence of this parasite in dogs, soil contamination and the resistance of eggs increase human exposure to the disease. Moreover, the difficulties of the control measures justify the need for alternative ones.
Aims: The objective of this study was to evaluate the in vitro ovicidal activity of fungi isolated from soils from public places in the city of Pelotas, Rio Grande do Sul, Brazil, on *Toxocara canis*.
Methods: Samples of soil from ten localities were inoculated onto Petri dishes with 2% water–agar (WA) that contained antibiotics, and incubated at 25 °C/21 days. Isolated fungi were tested in vitro for ovicidal activity, with five replicates. One mL of an embryonated *Toxocara canis* egg suspension (10^3 eggs) was poured over the fungal cultures after 10 days of growth. At intervals of 7, 14 and 21 days, 100 eggs were removed from each plaque and evaluated by optical microscopy.
Results: *Acremonium*, *Aspergillus*, *Bipolaris*, *Fusarium*, *Gliocladium*, *Mucor* and *Trichoderma* were isolated from the soil. A significant ovicidal type 3 effect was observed in *Trichoderma*, *Fusarium solani* complex and *Acremonium*. Those isolates from the genus *Trichoderma* showed their ovicidal effect on the 14th day of fungus–egg interaction. The other fungal genera tested showed a type 2 effect.
Conclusions: These results suggest that the use of *Trichoderma* and *Fusarium solani* complex in biological control of *T. canis* is promising; however, further studies should be performed.

Actividad ovicida fúngica sobre huevos de *Toxocara canis*

Palabras clave:
Toxocariasis
Nematodos
Suelo
Control biológico
Trichoderma spp.

RESUMEN

Antecedentes: La toxocariasis visceral es una zoonosis parasitaria causada por *Toxocara canis*. La prevalencia del parásito en perros, la contaminación del suelo y la resistencia de los huevos aumentan la exposición del ser humano a la infección. Además, las dificultades de las medidas de control justifican la búsqueda de otras alternativas.
Objetivos: El objetivo de este estudio fue evaluar la actividad ovicida *in vitro* frente a *T.* canis de hongos aislados del suelo de lugares públicos en la ciudad de Pelotas, Rio Grande do Sul, Brasil.
Métodos: Las muestras de suelo de 10 lugares diferentes se inocularon en placas de Petri con un 2% de agar/agua que contenía antibióticos y se incubaron a 25 °C durante 21 días. Se evaluó la actividad ovicida *in vitro* de los hongos aislados por quintuplicado. Después de 10 días de crecimiento, se vertió 1 ml (10^3 huevos) de una suspensión de huevos embrionarios de *T. canis* en los cultivos de hongos. A intervalos de 7, 14 y 21 días, se retiraron 100 huevos de cada placa y se evaluaron mediante microscopia óptica.
Resultados: Del suelo se aislaron especies de los géneros *Acremonium*, *Aspergillus*, *Bipolaris*, *Fusarium*, *Gliocladium*, *Mucor* y *Trichoderma*. Se observó un efecto ovicida significativo de tipo 3 en *Trichoderma*, el complejo de *Fusarium solani* y *Acremonium*. En hongos del grupo *Trichoderma* se evidenció un efecto ovicida el día 14 de la interacción hongo-huevo. Los otros géneros de hongos examinados mostraron un efecto de tipo 2.

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Conclusiones: Los resultados del presente estudio sugieren un uso prometedor de *Trichoderma* y *Fusarium solani* para el control biológico de *T. canis*, pero deben realizarse estudios adicionales.

Visceral toxocariasis, also called the syndrome of visceral larva migrans (VLM), is a parasitic zoonosis, and it is the result of the migration and persistence of helminthic larva in uncommon hosts.[3] Several species of helminthes can cause VLM; however, *Toxocara canis* is the nematode most frequently associated with the disease.[10] The high prevalence of this parasite in dogs, the observed frequent contamination of the environment, and the resistance of the parasite's eggs in the soil all increase human exposure to toxocariasis and make this disease a public health problem throughout the world.[28] The prevalence data regarding child toxocariasis from some countries demonstrate quite variable rates of infection.[14,31,37] In Brazil, studies have reported a prevalence that varies from 8.7% to 54.8%. [5,17,33] Studies evaluating contamination by the intestinal parasites of dogs in different Brazilian cities have shown that these animals are contaminated by various species of parasites with zoonotic potential.[22,23]

The difficulties inherent in implementing control measures, the high resistance of the eggs in the environment, and the problems inherent in chemical control justify the need for alternative measures that would help to decontaminate the soil.[2] Nematophagous fungi are natural enemies of nematodes. They possess activities directed to the parasitism of the eggs and larvae of the geohelminthes living outside of hosts and are increasingly popular candidates reducing the level of environmental contamination.[4] These fungi live in the organic matter of the soil, where they develop a parasitic or predator relationship with the nematodes and are classified as toxic, opportunistic (ovicidal), endoparasites and predators.[26] Those fungi parasiting eggs do not depend on the presence of nematodes in the soil for their survival. Due to this characteristic feature, they establish themselves more easily than predator fungi.[16] Their ovicidal activity is characterized by the penetration of haustorial hyphae into the egg shell through the pores of the vitelline layer; this alters the permeability of the shell and the expansion of its volume.[28] Among these fungi, *Pochonia chlamydosporia* and *Paecilomyces lilacinus* stand out and are extensively studied with regard to their ovicidal activity on *T. canis*.[1,6,18]

The aim of this study is to investigate and evaluate the ovicidal activity of fungi isolated from soil in southern Brazil on *T. canis* eggs.

Materials and methods

Collection of soil samples

Soil was collected from public areas in the municipality of Pelotas, State of Rio Grande do Sul, Brazil. The municipality is located at latitude 31°46′19″S and longitude 52°20′33″W. It is seven meters above sea level, and has a damp, subtropical climate with an annual average temperature of 17.5 °C, 1379 mm of rain per year, and an average relative humidity of 80%.[21] The areas selected for the soil collection (10 localities; 3 equidistant places per locality) were chosen based on previous works that had been performed in the region and had shown soil contamination with *Toxocara* eggs.[19,38] Samples of approximately 500 g of soil were obtained at a depth of 10 cm. The dead leaves and other residual organic matter of the top layer were ignored. After the collection, the samples were packed in plastic bags, identified, and immediately transported to the Mycology Laboratory of the Biology Institute/Federal University of Pelotas (UFPel) for processing.

Collection of T. canis *eggs*

The eggs were obtained directly from the uterine tubes of adult females and were washed 10 times with distilled sterile water and centrifuged at 1000 rpm for 5 min. Next, they were incubated at 25 °C for 14 days in a solution containing formalin at 0.05%, streptomycin sulfate at 0.05%, and chloramphenicol at 0.01%. The embryonated eggs had been previously analyzed morphologically to verify their integrity.

Isolation of the fungi from the soil

The technique for the isolation of fungi was based on that of Duddington [12] and adapted for the research on fungi with ovicidal activity described by Gortari et al.[20] The 2% water/agar (WA), which contained added streptomycin 5 mg/1 L and chloramphenicol 5 mg/1 L, was inoculated at the surface with a 1 ml suspension of *T. canis* eggs (approximately 10^3 eggs) and immediately seeded with 0.5 g of the soil, previously homogenized, in the form of a cross. The Petri dishes were incubated at 25 °C and observed daily for 21 days. The identification of the fungi was based on macro- and micro-morphological characteristics[9,11] and determined to the lowest possible taxonomic level.

In vitro activity of the fungal isolates

The Petri dishes of isolates that contained Potato dextrose agar (PDA) were selected and incubated at 25 °C for 10 days. From these cultures, 4 mm disks were transferred to Petri dishes containing 2% WA. All the dishes were incubated at 25 °C for 10 days. A 1 mL suspension of embryonated *T. canis* eggs (10^3/mL) was poured over each of the fungal cultures, as well as over the surface of Petri dishes containing only 2% WA (without fungus) as a control. Five replicates were performed for each fungal isolate analyzed. At intervals of 7, 14 and 21 days, 100 eggs were removed from each dish, according to the technique described by Araújo et al.,[1] colored with Aman-blue at 1% and evaluated in microscopy of light (object lense 40×), according to the parameters established by Lysek[24]: effect type 1, physiological, biochemical effect, without morphological damage to the egg shell, on which hyphae can be observed adhered to the shell; effect type 2, lithic effect with alterations in the morphology of the shell and embryo of the egg, without penetration of the hyphae through the shell; effect type 3, lithic effect, with morphological alteration of the shell and the embryo, as well as penetration of the hyphae and internal colonization of the egg.

Statistical analysis

The data obtained were submitted to the non-parametric Friedman test with a significance level of 1%. The fungi that differ their effects by Friedman test also underwent regression analysis up to the second order regarding the dates of assessment. In regression analysis, the choice model was based on the significance of linear and quadratic coefficient by using the Student test at 5% probability. The analyses were performed using the SAS software package.[32]

Table 1
Percentages and standard deviation of ovicide activity of the fungi *Acremonium*, *Fusarium solani* complex, *Trichoderma* and control group, on *Toxocara canis* eggs at 7, 14 and 21 days of fungus–egg interaction.

	Effect 1 (%)[a]	Effect 2 (%)[b]	Effect 3 (%)[c]
Effects at 7 days			
Acremonium	$27.4^{A} \pm 3.3$	$0^{A} \pm 0$	$0^{A} \pm 0$
Fusarium solani complex	$52.0^{B} \pm 3.8$	$0^{A} \pm 0$	$0^{A} \pm 0$
Trichoderma	$64.0^{C} \pm 2.6$	$10.6^{B} \pm 5.9$	$0.2^{A} \pm 0.4$
Control	$0^{D} \pm 0$	$0^{A} \pm 0$	$0^{A} \pm 0$
Effects at 14 days			
Acremonium	$49.0^{A} \pm 1.6$	$4.8^{A} \pm 2.7$	$0^{A} \pm 0$
Fusarium solani complex	$59.6^{B} \pm 1.5$	$7.0^{A} \pm 2.0$	$0^{A} \pm 0$
Trichoderma	$35.2^{C} \pm 3.9$	$41.2^{B} \pm 6.4$	$3.4^{B} \pm 1.1$
Control	$0^{D} \pm 0$	$0^{C} \pm 0$	$0^{A} \pm 0$
Acremonium	$51.6^{A} \pm 3.7$	$30.2^{A} \pm 3.0$	$1.8^{A} \pm 0.8$
Fusarium solani complex	$49.0^{A} \pm 4.3$	$26.0^{A} \pm 3.5$	$4.6^{B} \pm 1.1$
Trichoderma	$30.8^{B} \pm 3.6$	$53.4^{B} \pm 5.1$	$7.8^{C} \pm 1.3$
Control	$0^{C} \pm 0$	$0^{C} \pm 0$	$0^{D} \pm 0$

Percentage followed by a different capital letter in the column statistically differs ($p < 0.01$) by the Friedman test.

[a] Effect type 1, physiological, biochemical effect, without morphological damage to the egg shell, on which hyphae can be observed adhered to the shell.

[b] Effect type 2, lithic effect with alterations in the morphology of the shell and embryo of the egg, without penetration of the hyphae through the shell.

[c] Effect type 3, lithic effect, with morphological alteration of the shell and the embryo, as well as penetration of the hyphae and internal colonization of the egg.

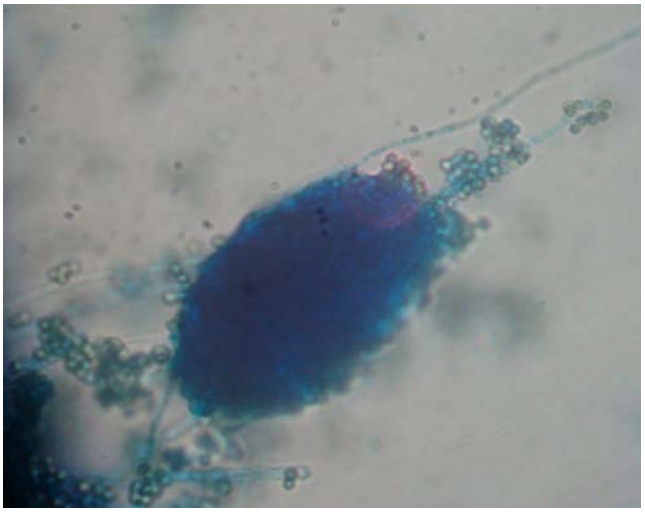

Fig. 1. Effect type 3 of the insulator *Trichoderma* spp. on *Toxocara canis* eggs on the 14th day of interaction. The image shows the adherence of conidia and penetration of hyphae in the cuticle with the destruction of the egg. Optical microscopy (object lense 40×).

Results

Isolates from the genera *Aspergillus, Acremonium, Bipolaris, Fusarium oxysporum* complex*, Fusarium solani* complex*, Gliocladium, Mucor and Trichoderma* were recovered from the soil. All the fungal isolates showed some degree of fungus–egg interaction. However, the isolates of *Acremonium*, *Fusarium solani* complex and *Trichoderma* presented a significant ovicidal effect type 3 on the embryonated *T. canis* eggs when submitted to the non-parametric Friedman test. The average results for the type 1, 2, and 3 effects at 7, 14, and 21 days for the groups treated with the fungi *Acremonium*, *Fusarium solani* complex, and *Trichoderma* and the control group are detailed in Table 1. In the control group, no fungal growth was detected. The ovicidal activity of the *Trichoderma* isolate was observed on the 14th day of interaction (Fig. 1). With the *Acremonium* and *Fusarium solani* complex isolates, the same effect was observed on the 21st day.

Table 2
Regression equations of the effects of the fungi *Trichoderma* spp., *Fusarium solani* complex and *Acremonium* spp. on *Toxocara canis* eggs.

Fungal isolate		R^2 (%)	p
Trichoderma	E1 $\hat{Y} = 76.533 - 2371x$	79.49	<0.0001
	E2 $\hat{Y} = -7733 + 3057x$	86.88	<0.0001
	E3 $\hat{Y} = -3.80 + 0.543x$	91.16	<0.0001
	NE $\hat{Y} = 35 - 1.229x$	79.49	<0.0001
Fusarium solani complex	E1 $\hat{Y} = 26.200 + 4.986x - 0.186x^2$	67.89	0.0004
	E2 $\hat{Y} = -15.00 + 1.857x$	90.08	<0.0001
	E3 $\hat{Y} = -3.067 + 0.329x$	69.85	<0.0001
	NE $\hat{Y} = 61.533 - 1.971x$	93.04	<0.0001
Acremonium	E1 $\hat{Y} = 18.467 + 1.729x$	78.15	<0.0001
	E2 $\hat{Y} = -18.533 + 2.157x$	84.47	<0.0001
	E3 $\hat{Y} = -1.20 + 0.129x$	59.56	0.0008
	NE $\hat{Y} = 101.267 - 4.014x$	98.47	<0.0001

E1, effect type 1; E2, effect type 2; E3, effect type 3; NE, no effect

The regression analysis showed variations in effects 1, 2 and 3 for each fungal strain evaluated in connection to the time of exposure of *T. canis* eggs to the fungi (Table 2). The analysis revealed that independently of the fungus, type 2 and 3 effects presented increasing linear response. In *Trichoderma* it was observed that type 1 effect decreased linearly (2.371 every day). When evaluated type 2 and 3 effects, it was verified that, although both had evidenced increasing linear response, they differ ($p < 0.0001$): an increase of 3.057 and 0.543 every day, respectively, was observed. The type 1 effect of *Fusarium* complex *solani* presented a quadratic response with the highest value on the 13th day after the initiation of treatment. Yet the type 2 effects showed increase of linear response with increase of 1.857 every day, as well as type 3 effect which increased 0.32 every day. Finally, the type 1 and 2 effect of *Acremonium* spp. demonstrated increasing linear response and similar, increasing 1.729 and 2.157 each day, respectively ($p > 0.05$). Nevertheless, the type 3 effect showed different response ($p > 0.05$) with an increase of 0.129 every day.

Although the remaining evaluated fungi did not show a significant type 3 effect, a type 2 effect was observed on the 21st day in the *Aspergillus* (55.2%), *Bipolaris* (52.4%), *Fusarium* complex *oxysporum* (46.8%), *Mucor* (42.0%) and *Gliocladium* (39.2%) isolates.

Discussion

This study is the first that researches parasitic fungi for reduction of *T. canis* eggs in the soil of the southern region of Brazil. Isolates from *Trichoderma*, *Fusarium solani* complex and *Acremonium* were notable for their promising ovicidal activities. According to Lysek,[24] a fungus with ovicidal potential is one that demonstrates a large lithic effect, causing morphological alteration of the shell and embryo with penetration of hyphae and internal colonization of the egg (type 3 effect). Among the fungi evaluated, *Trichoderma* spp. stood out, as it presented a significant type 3 effect at the 14th day of fungus–egg interaction. This result is relevant because Overgaauw[29] reported that *T. canis* eggs become infectious after approximately 2–6 weeks. Hence, this fungus could render the eggs inactive and thus reduce the level of environmental contamination. Several studies have evaluated the ovicidal action of different fungi on *T. canis* eggs. [1,7,8,18,20] Only Ciarmela et al.[8] evaluated the activity of *Trichoderma harzianum*. This genus has been extensively researched in the biological control of numerous phytopathogenic fungi[15,25,30] and in the control of phytonematodes.[16,35,36] Surveys have shown that *Trichoderma* spp. have the capacity for parasiting the eggs of different nematode species from the

species *Meloidogyne exigua*,[16] *Meloidogyne incognita*,[13,34] *Meloidogyne arenaria*[39] and *Meloidogyne javanica*.[35,36] Mechanisms that have been suggested to explain the activity of *Trichoderma* against phytopathogenic fungi include antibiosis, competition, microparatisism and production of enzymes (chitinases, glucanases and proteases). All of these mechanisms, with the exception of competition, could potentially be involved in the process of biological nematode control.[35] Santin,[34] when assessing the potential of *Trichoderma* on *M. incognita* control, suggested that the mechanisms used by the fungus consist of the production of both inhibiting volatile metabolites and lithic enzymes that degrade the chitin of the eggs. Morton[27] stated that the chitinolytical activity is likely the most relevant for the lesion on the sheath of the egg. This suggests that the ovicidal activity of the observed *Trichoderma* spp. on *T. canis* eggs in the current study may be due to some of the aforementioned mechanisms. However, further research is needed to determine the exact ovicidal mechanism used by the fungus. Although our study has not identified the species of *Trichoderma* isolated, our results differ from Ciarmela et al.,[8] who found that *T. harzianum* does not affect the viability of *T. canis* eggs.

In Argentina, Ciarmela et al.[7,8] evaluated ovicidal activity of isolated fungi in soil of public localities in the city of La Plata and showed the activity of *Fusarium pallidoroseum*, *Fusarium oxysporum*, *Fusarium sulphureum* and *Fusarium moniliforme* on *T. canis* eggs. In a similar study, Gortari et al.[20] demonstrated the efficiency of various fungal genera on eggs of the same parasite, among which were *Acremonium*, *Aspergillus*, *Fusarium*, *Mucor*, *Paecilomyces*, and others. The observed ovicidal effect of the genus *Fusarium* in our study and in those of Ciarmela et al.[7,8] and Gortari et al.[20] indicate the potential of these fungi as agents of biological control of helminthic eggs. Larger investigations are necessary.

The most studied nematophagous fungi, *Paecilomyces lilacinus* and *Pochonia chlamydosporia*, stand out for their ovicidal activity. *In vitro* studies have shown the efficiency of these fungi on embryonated *T. canis* eggs.[1,6,18] Although they are the species most often cited as ovicides, *Pochonia chlamydosporia* and *Paecilomyces lilacinus* were not isolated from the soil in this study.

Conclusion

The results of this study verify the presence of fungi parasitic to *T. canis* eggs in the soil from the Southern region of Brazil and highlight the ovicidal activity of *Trichoderma* and *Fusarium solani* complex. However, more studies to prove the biological potential of fungi evaluated, as well as their mechanisms of ovicidal action, are required. We believe that further research aimed at the search for autochthonous fungi with nematophagous potential is important, since the adaptation of the fungus to different regional conditions is a key factor to the biological control of parasites.

Conflict of interest

The authors declare that there is no conflict of interest.

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