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Original article

Effect of *Paecilomyces lilacinus*, *Trichoderma harzianum* and *Trichoderma virens* fungal extracts on the hatchability of *Ancylostoma* eggs

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ABSTRACT

Background: *Ancylostoma* species have demanded attention due to their zoonotic potential. The use of anthelmintics is the usual method to prevent environmental contamination by *Ancylostoma* eggs and larvae. Nematophagous fungi have been widely used in their biological control due to the fungus ability to capture and digest free nematode forms.
Aims: The aim of this study was to evaluate the effect of four different fungal extracts of *Paecilomyces lilacinus* ($n = 2$), *Trichoderma harzianum* ($n = 1$) and *Trichoderma virens* ($n = 1$) isolates on the hatchability of *Ancylostoma* eggs.
Methods: Fungal extracts consisted of fungal broth culture supernatant without filtration (crude extract) and filtered broth (filtered extract), macerated mycelium (crude macerate), and macerated mycelium submitted to filtration (filtered macerate). The *Ancylostoma* eggs were obtained from the feces of naturally infected dogs. *In vitro* assays were performed in five replicates and consisted of four treatments and one control group.
Results: The activity of the fungal extracts of each evaluated fungus differed ($p < 0.05$) from those of the control group, showing significant ovicidal activity. The hatching of the eggs suffered reduction percentages of 68.43% and 47.05% with *P. lilacinus*, and 56.43% with *T. harzianum*, when crude macerate extract was used. The reduction with the macerate extract of *T. virens* was slightly lower (52.25%) than that for the filtered macerate (53.64%).
Conclusions: The results showed that all extracts were effective in reducing the hatchability of *Ancylostoma* eggs. The ovicidal effect observed is likely to have been caused by the action of hydrolytic enzymes secreted by the fungi.

Efecto de los extractos de los hongos *Paecilomyces lilacinus*, *Trichoderma harzianum* y *Trichoderma virens* en la eclosionabilidad de huevos de *Ancylostoma*

Palabras clave:
Ovicida
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RESUMEN

Antecedentes: Las especies del género *Ancylostoma* son de gran importancia debido a su potencial zoonótico. El uso de antihelmínticos es el método habitual en la prevención de la contaminación ambiental por huevos y larvas del género *Ancylostoma*. Los hongos nematófagos se utilizan ampliamente en el control biológico de aquellos, debido a su capacidad de capturar y digerir nematodos libres.

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Objetivo: El objetivo del estudio fue evaluar el efecto de cuatro extractos diferentes de hongos (*Paecilomyces lilacinus* [n = 2], *Trichoderma harzianum* [n = 1] y *Trichoderma virens* [n = 1]) en la eclosionabilidad de huevos de especies de *Ancylostoma*.
Métodos: Los extractos de hongos constaban del sobrenadante del cultivo en caldo fúngico sin filtración (extracto crudo) y caldo filtrado (extracto filtrado), micelio macerado (macerado crudo) y micelio macerado sometido a filtración (macerado filtrado). Los huevos de *Ancylostoma* se obtuvieron a partir de heces de perros infectados de manera natural. Se realizaron cinco repeticiones de los ensayos in vitro con cuatro tratamientos y un grupo control.
Resultados: La actividad de los extractos fúngicos de cada hongo evaluado difiere ($p < 0,05$) de la de aquellos del grupo control, lo que demuestra una actividad ovicida significativa. Con el extracto crudo macerado, la reducción de la eclosión mostró porcentajes del 68,43 y el 47,05% en el caso de *P. lilacinus* y del 56,43% para el caso de *T. harzianum.* El porcentaje de reducción en el uso del macerado crudo en *T. virens* fue del 52,25%, algo inferior respecto al macerado filtrado (53,64%).
Conclusiones: Los resultados mostraron que todos los extractos fueron eficaces en la reducción de la eclosionabilidad de huevos de *Ancylostoma*. Es probable que el efecto ovicida observado haya sido causado por la acción de enzimas hidrolíticas secretadas por los hongos.

Nematophagous fungi comprise different types of fungi and are the major nematode natural enemies, so, they have been used in their biological control due to the fungus ability to capture and digest free nematode forms.[10,11]

Ancylostoma caninum and *Ancylostoma braziliense* have demanded considerable attention due to their zoonotic potential, which is directly related to soil contamination with the feces of infected animals.[7] Although the use of anthelmintics is the usual method to prevent environmental contamination by *Ancylostoma* eggs and larvae, the development and implementation of alternative measures for control of geohelminths are crucial to reduce environmental contamination by the infective forms of this parasite.[7] Furthermore, the increase in the number of reports of nematodes' resistance to the different drugs available and the growing trend toward using products that do not harm the environment stimulate the search for alternative methods.[10] In this context, nematophagous fungi can be used in combination when the environment is already contaminated.[7]

Ovicidal or opportunistic fungi such as *Paecilomyces lilacinus* and *Pochonia chlamydosporia* have been used successfully for the *in vitro* control of gastrointestinal helminth eggs from animals.[1,3,7] Studies have shown that the mechanism of infection of these fungi can be mechanical, enzymatic, or a combination of both.[2] However, in the last decade, the identification of numerous extracellular enzymes has confirmed their involvement as important virulence factors associated with the infection process.[11] A significant enzymatic activity has been reported when filtered cultures of *Paecilomyces lilacinus* and *Trichoderma* were used on phytonematodes,[2,12,16] or when the crude enzymatic extract of *Pochonia chlamydosporia* and *Duddingtonia flagrans* were used on eggs and larvae of animal nematodes.[4–6]

However, enzymatic extracts of *Paecilomyces lilacinus* and *Trichoderma* have not yet been tested on geohelminths eggs, such as *Ancylostoma*, which hatch for a short period of time in the environment. The aim of this paper was to evaluate the *in vitro* action of four different *Paecilomyces lilacinus*, *Trichoderma harzianum* and *Trichoderma virens* fungal extracts on *Ancylostoma* eggs.

Material and methods

Fungal cultures

Four fungal isolates were used – CG193 *Paecilomyces lilacinus* and CG502 *Trichoderma harzianum* provided by Cenargen (Embrapa Genetic Resources and Biotechnology), MICLAB009 *Paecilomyces lilacinus* and MICLAB008 *Trichoderma virens* obtained from the collection of fungi of the Mycology Laboratory, Biology Institute, Federal University of Pelotas, Brazil properly identified by DNA sequencing. The cultures kept in test tubes containing potato agar (PDA) at 4 °C were subcultured on Petri dishes with PDA and incubated at 25 °C for 10 days. Then 4 mm fungal culture disks of each isolate were transferred to Erlenmeyer flasks containing 150 ml minimal medium broth [glucose (1.8 g/l); NH_4NO_3 (0.4 g/l); $MgSO_4$ 7 H_2O (0.12 g/l); Na_2HPO_4 7 H_2O (3.18 g/l), KH_2PO_4 (0.26 g/l), yeast extract (0.3 g/l) and gelatin for bacteriological use (2 g/l)]. The flasks were incubated at 28 °C on a rotary shaker at 120 rpm for five days.[6]

Preparation of fungal extracts

Four different extracts were obtained from the cultures in minimum medium broth: crude extract (CE), consisting of supernatant broth; filtered extract (FE) obtained by filtering the supernatant broth on filter paper (Whatman N°1); crude macerate (CM), obtained by macerating mycelium in three liquid nitrogen baths until a powdery consistency was obtained, subsequently resuspended in the supernatant broth; and filtered macerate (FM), obtained in the same manner as crude macerate, but subjected to filtration through filter paper (Whatman N°1). All extracts were prepared and used on the same day.

Fecal samples

A 500 g fresh feces pool from naturally infected dogs of the Pelotas City Kennel was collected every day during the experiment. Initially, the feces were diluted and macerated in warm water and then filtered through 1 mm, 105 μm, 55 μm and 25 μm sieves. The residue of the last sieve was washed in distilled water and the suspension centrifuged at 3000 rpm for five minutes, the supernatant was then discarded, and the pellet was suspended in supersaturated saline and centrifuged again under the same conditions. Following, the supernatant was filtered through a 25 μm sieve and the eggs collected by distilled water wash, counted in a Neubauer chamber and used on the same day.

Experimental assays

The *in vitro* assays consisted of four treatments and a control group. Four ml of CE, FE, CM and FM fungal extracts were poured

into 60 mm × 15 mm Petri dishes. Then 1 ml of a suspension containing 10^3 *Ancylostoma* eggs was added. The control group dishes were poured a suspension containing 10^3 *Ancylostoma* eggs in 4 ml minimum medium broth. All dishes were incubated at 25 °C for 24 h. Each treatment consisted of five replicates. After 24 h, the reading was performed by a stereoscope and the total number of *Ancylostoma* larvae present in the treated and control groups was estimated.

Statistical analysis

The experimental design was completely randomized with five treatments and five replicates. As the response variable showed no normality, the data were subjected to the nonparametric Kruskal–Wallis test; when differences between treatments were found, the means were compared by the Bonferroni test. The analyses were performed with the aid of SAS statistical software, assuming a 5% probability. The mean reduction percentage of larvae was calculated through the following equation[4,5]

$$\text{Reduction \%} = \frac{(\text{average of larvae in control group} - \text{average of larvae in treated group})}{\text{average of larvae in control group}} \times 100$$

Results

After a 24-h-interaction period, the fungal extracts (CE, FE, CM and FM) evaluated were observed to reduce the *Ancylostoma* hatchability to some extent, as compared to control (Table 1). Statistical analysis showed differences ($p < 0.05$) in the number of larvae between the fungal extracts of each fungus and the control group. Moreover, it showed that CE, FE, CM and FM did not present the same pattern for each fungus tested (Table 1). However, when the hatchability reduction percentage of *Ancylostoma* eggs was analyzed, it was evidenced that the greatest hatchability reduction occurred when CM was used, and 68.43% MICLAB009 *P. lilacinus*, 47.05% CG193 *P. lilacinus* and 56.43% CG502 *T. harzianum* reduction percentages were observed. The percentage CM reduction (52.25%) was slightly lower than that of FM (53.64%) only for the *T. virens* isolate.

Table 1
Mean number of larvae and hatching reduction percentages of *Ancylostoma* eggs subjected to treatment with different fungal extracts of *P. lilacinus* ($n = 2$), *T. harzianum* ($n = 1$) and *T. virens* ($n = 1$) in a 24-h-period.

Fungal extracts	Mean number of larvae	Reduction percentage (%)
P. lilacinus (CG 193)		
CM	369.2[c]	47.03
FM	524.2[b]	24.79
CE	522.2[b]	25.07
FE	385.2[c]	44.73
Control	697[a]	
P. lilacinus (MICLAB 009)		
CM	119.4[c]	68.32
FM	208.2[b]	44.77
CE	152.6[b]	59.52
FE	215.2[b]	42.91
Control	377[a]	
T. virens (MICLAB 008)		
CM	446[c]	52.24
FM	433.6[c]	53.57
CE	472[c]	49.46
FE	527[b]	43.57
Control	934[a]	
T. harzianum (CG 502)		
CM	159.4[c]	56.35
FM	204.8[c]	43.92
CE	269.8[b]	26.12
FE	199.8[c]	45.29
Control	365.2[a]	

Means followed by different letters in the column differ statistically ($p < 0.05$).
CM, crude macerate; FM, filtered macerate; CE, crude extract; FE, filtered extract.

Discussion

Nematophagous fungi have been widely used for biological control because of their ability to capture and infect nematodes through enzymatic action.[11,12]

The results of this study show the ovicidal activity of the evaluated fungi on *Ancylostoma* eggs and suggest that the activity may be due to the action of hydrolytic enzymes. Furthermore, the use of enzymatic extracts of the fungi significantly reduced *Ancylostoma* eggs hatching after a 24-h-exposure period. This ovicidal activity could be an advantage, since it would allow the employment of fungus enzymatic extracts on geo-helminth eggs that hatch in a short period of time in the environment, as *Ancylostoma*, that hatch in approximately 5 days.[17]

Although the pathogenic mechanisms of nematophagous fungi are not fully understood, evidence shows that extracellular hydrolytic enzymes, including proteases, collagenases and chitinases, may be involved in the digestion and penetration of the cuticle of nematodes.[11,14,20] Kahn et al.[12] upon evaluating the effect of *P. lilacinus* on *Meloidogyne javanica* eggs, showed that the disintegration of the vitelline, lipid and chitinous layers of the eggs was caused solely by enzymatic degradation of proteases and chitinases. *P. lilacinus* serine protease was responsible for the damage to the shell and vacuolization of *Meloidogyne hapla* eggs, playing a key role in the fungus pathogenicity.[2] In addition to serine protease, chitinase activity has also been observed in *P. lilacinus* culture supernatant.[12]

In this study, the two *P. lilacinus* isolates evaluated were able to reduce the hatching of *Ancylostoma* eggs, showing significant ovicidal activity. Previous studies have demonstrated the ovicidal effect of this fungal species on *Toxocara canis*[1] and *Taenia saginata*.[3]

Studies have shown the potential of *Trichoderma* in the biological control of different *Meloidogyne* species.[8,16] In 2013, Filho et al.,[9] upon evaluating the ovicidal ability of fungi isolated from Brazilian soil on *Toxocara canis* eggs, identified a *Trichoderma* isolate with promising ovicidal activity.

The use of different enzymatic extracts of *T. harzianum* and *T. virens* in this study demonstrates the ovicidal potential of these fungal species on *Ancylostoma* eggs. Morton et al.[14] and Romão-Dumaresq et al.[15] argue that the chitinolytic activity of these fungi is probably the most relevant effect to the egg sheath injury.

Although the authors of this study have not identified and purified the enzymes present in the fungi extracts evaluated, it is believed that the ovicidal effect observed resulted from the enzymatic degradation of proteases and chitinases. Since the eggs of parasites of the Phylum Nematoda may have one to three layers, an inner lipoproteic layer, an intermediate chitinous one and an outer vitelline one, these layers are likely to be susceptible to these enzymes. Previous studies demonstrated the lytic effect of purified proteases and chitinases on *Haemonchus contortus* eggs.[13] However, the development of future studies aimed at the identification and characterization of enzymes and their activities on animal pathogen helminth eggs is essential for the continuity and use of these fungi in the biological control of parasites.

Even though most studies have assessed filtered cultures of fungi on eggs of phytonematodes and gastrointestinal nematodes

of domestic animals,[2,4–6,12,16] we opted to test different fungal extracts involving filtered and macerated cultures. It was observed that, regardless of the fungal extract, there always was some level of ovicidal activity. However, the highest hatchability reduction percentage was observed with crude macerate extract, particularly that from MICLAB009 *P. lilacinus* isolate. The authors suggest that such activity could result from the presence of intracellular enzymes released during the maceration process which, together with the action of extracellular enzymes, would increase the fungus efficiency. However, this can only be confirmed with the development of studies that evaluate the isolated and combined action of the enzymes involved. On the other hand, some studies have evaluated the enzymatic activity of filtered cultures or purified enzymes of nematophagous fungi on larvae and eggs of gastrointestinal helminths of animals. When the results of these studies were evaluated, it was observed that, upon testing the enzymatic activity of a serine protease isolated from *Monacrosporium thaumasium*, the reduction in the number of *Angiostrongylos vasorum* larvae was only 23.9%.[18] Nevertheless, when *D. flagrans* crude enzymatic extract was used on larvae of the same nematode, the reduction percentage reached 71.3%,[6] suggesting that the combination of hydrolytic enzymes increases the ovicidal activity. Similarly, it was found in other studies using crude enzyme extracts of *Pochonia chlamydosporia* on Cyathostominae[4] and *Ancylostoma* eggs[6] an egg hatchability reduction of 72.8% and 76.8%, respectively, which is similar to that reported by us in the present study. In a previous research, Huang et al.[11] found that the synergism of proteases and chitinases of *P. lilacinus* was able to significantly reduce the development and hatching of *M. javanica* eggs. Likewise, Tikhonov et al.[19] reported that the combined action of proteases and chitinases destroys the lipid layers of the egg, causes hydrolysis of chitin and alters the vitelline layer.

Conclusion

The use of enzymatic extracts of the fungi *P. lilacinus*, *T. harzianum* and *T. virens* significantly reduces *Ancylostoma* eggs hatching after a 24-h-exposure period. Thus, these fungi are, together with other known nematophagous fungi, promising biocontrol agents of geohelminths in the environment. Yet, additional studies are needed so that the molecules responsible for the observed effects can be identified and characterized.

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Conflict of interest

The authors declare that there is no conflict of interest.

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