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Original article

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

Bianca Delgado Menezes Hofstätter[a], Anelise Oliveira da Silva Fonseca[a], Fernando de Souza Maia Filho[a], Julia de Souza Silveira[a], Beatriz Maroneze Persici[a], Luciana Pötter[b], Andressa Silveira[b], Zaida Inês Antoniolli[b], Daniela Isabel Brayer Pereira[a,*]

[a] *Universidade Federal de Pelotas (UFPel), Instituto de Biología, Departamento de Microbiologia e Parasitologia, Pelotas, Rio Grande do Sul, Brazil*
[b] *Universidade Federal de Santa Maria (UFSM), Centro de Ciências Rurais, Departamento de Zootecnia, Santa Maria, Rio Grande do Sul, Brasil*

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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.

* Corresponding author.
E-mail address: danielabrayer@gmail.com (D.I. Brayer Pereira).

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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

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