Original article

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

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A B S T R A C T

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 oviducal 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 oviducal effect observed is likely to have been caused by the action of hydrolytic enzymes secreted by the fungi.

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

R E S U M E N

Antecedentes: Las especies del género Ancylostoma son de gran importancia debido a su potencial zoonótico. El uso de antihelminticos 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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Palabras clave:
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Hongos nematófagos
Enzimas

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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.  

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. 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 is to reduce environmental contamination by the infective forms of this parasite. 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. In this context, nematophagous fungi can be used in combination when the environment is already contaminated.  

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. Studies have shown that the mechanism of infection of these fungi can be mechanical, enzymatic, or a combination of both. However, in the last decade, the identification of numerous extracellular enzymes has confirmed their involvement as important virulence factors associated with the infection process. A significant enzymatic activity has been reported when filtered cultures of Paecilomyces lilacinus and Trichoderma were used on phytomematodes or when the crude enzymatic extract of Pochonia chlamydosporia and Duddingtonia flagrans were used on eggs and larvae of animal nematodes.  

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 virnes 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 virnes 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); NH₄NO₃ (0.4 g/l); MgSO₄ 7 H₂O (0.12 g/l); Na₂HPO₄ 7 H₂O (3.18 g/l), KH₂PO₄ (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.

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 the statistical software, assuming a 5% probability. The mean reduction percentage of larvae was calculated through the following equation:

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

**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% MCLAB009 _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.

**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 fung- enzolic extracts on geo-helmint 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.\(^{11}\) upon evaluating the effect of _P. lilacinus_ on _Meloidogyne javanica_ eggs, showed that the dis-integration 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.\(^{8,10}\) 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.\(^{12}\) 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 phytomonad and gastrointestinal nematodes

**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.

<table>
<thead>
<tr>
<th>Fungal extracts</th>
<th>Mean number of larvae</th>
<th>Reduction percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. lilacinus (CG 193)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>369.2(^{+})</td>
<td>47.03</td>
</tr>
<tr>
<td>FM</td>
<td>524.2(^{+})</td>
<td>24.79</td>
</tr>
<tr>
<td>CE</td>
<td>522.2(^{+})</td>
<td>25.07</td>
</tr>
<tr>
<td>FE</td>
<td>385.2(^{+})</td>
<td>44.73</td>
</tr>
<tr>
<td>Control</td>
<td>697(^{+})</td>
<td></td>
</tr>
<tr>
<td><strong>P. lilacinus (MCLAB 009)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>119.4(^{+})</td>
<td>68.32</td>
</tr>
<tr>
<td>FM</td>
<td>208.2(^{+})</td>
<td>44.77</td>
</tr>
<tr>
<td>CE</td>
<td>152.6(^{+})</td>
<td>59.52</td>
</tr>
<tr>
<td>FE</td>
<td>215.2(^{+})</td>
<td>42.91</td>
</tr>
<tr>
<td>Control</td>
<td>377(^{+})</td>
<td></td>
</tr>
<tr>
<td><strong>T. virens (MCLAB 008)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>446(^{+})</td>
<td>52.24</td>
</tr>
<tr>
<td>FM</td>
<td>433.6(^{+})</td>
<td>53.57</td>
</tr>
<tr>
<td>CE</td>
<td>472(^{+})</td>
<td>49.46</td>
</tr>
<tr>
<td>FE</td>
<td>527(^{+})</td>
<td>43.57</td>
</tr>
<tr>
<td>Control</td>
<td>934(^{+})</td>
<td></td>
</tr>
<tr>
<td><strong>T. harzianum (CG 502)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>159.4(^{+})</td>
<td>56.35</td>
</tr>
<tr>
<td>FM</td>
<td>204.8(^{+})</td>
<td>43.92</td>
</tr>
<tr>
<td>CE</td>
<td>269.8(^{+})</td>
<td>26.12</td>
</tr>
<tr>
<td>FE</td>
<td>119.8(^{+})</td>
<td>45.29</td>
</tr>
<tr>
<td>Control</td>
<td>365.2(^{+})</td>
<td></td>
</tr>
</tbody>
</table>

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.
of domestic animals.\textsuperscript{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 oviocidal activity. However, the highest hatchability reduction percentage was observed with crude macerate extract, particularly that from MICLAB009 \textit{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 \textit{Monacrosporium thamaisium}, the reduction in the number of \textit{Angiostrongylus vasorum} larvae was only 23.9%.\textsuperscript{18} Nevertheless, when \textit{D. flagrans} crude enzymatic extract was used on larvae of the same nematode, the reduction percentage reached 71.3%,\textsuperscript{6} suggesting that the combination of hydrolytic enzymes increases the oviocidal activity. Similarly, it was found in other studies using crude enzyme extracts of \textit{Pochonia chlamydosporia} on Cyathostominae\textsuperscript{1} and \textit{Ancylostoma eggs}\textsuperscript{2} 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.\textsuperscript{17} found that the synergism of proteases and chitinases of \textit{P. lilacinus} was able to significantly reduce the development and hatching of \textit{M. javanica} eggs. Likewise, Tikhonov et al.\textsuperscript{18} 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 \textit{P. lilacinus}, \textit{T. harzianum} and \textit{T. virens} significantly reduces \textit{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.

**References**