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Research Brief

Influence of the preservation period in silica-gel on the predatory activity of the isolates of *Duddingtonia flagrans* on infective larvae of cyathostomins (Nematoda: Cyathostominae)

Fabio Ribeiro Braga [a,*], Jackson Victor Araújo [a,1], Juliana Milani Araujo [a], Alexandre de Oliveira Tavela [a], Sebastião Rodrigo Ferreira [a], Filippe E. Freitas Soares [b], Laércio dos Anjos Benjamin [a], Luiza Neme Frassy [a]

[a] Departamento de Veterinária, Universidade Federal de Viçosa, Viçosa-MG, CEP 36570-000 Vicosa, Brazil
[b] Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, Viçosa-MG, CEP 3657-000 Vicosa, Brazil

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ABSTRACT

The continued maintenance of nematophagous fungi predatory activity under laboratory conditions is one of the basic requirements for a successful biological control. The purpose of this study was to evaluate the influence of time on the preservation of the fungus *Duddingtonia flagrans* (AC001 and CG722) stored in silica-gel for 7 years and their subsequent predatory activity on cyathostomin L_3 larvae in 2% water-agar medium (2% WA). Samples of the isolates AC001 and CG722, originating from vials containing grains of silica-gel sterilized and stored for 7 years, were used. After obtaining fungal conidia, the predation test was conducted over 7 days on the surface of 9.0 cm Petri dishes filled with 2% WA. In the treated groups each Petri dish contained 500 cyathostomin L_3 and conidia of fungal isolates in 2% WA. In the control group (without fungi) the plates contained 500 L_3 in 2% WA. The experimental results showed that isolated AC001 and CG722 were efficient in preying on cyathostomin L_3 ($p < 0.01$) compared to control (without fungus). However, no difference was observed ($p > 0.01$) in the predatory activity of the fungal isolates tested. Comparing the groups, there was a significant reductions of cyathostomin L_3 ($p < 0.01$) of 88.6% and 78.4% on average recovered from the groups treated with the isolates AC001 and CG722, respectively, after 7 days. The results of this test showed that the fungus *D. flagrans* (AC001 and CG722) stored in silica-gel for at least 7 years maintained its predatory activity on cyathostomin L_3.

1. Introduction

Horses host a wide variety of helminths, and cyathostomins are one of the most important (Love and Duncan, 1992). These helminths are present in pastures throughout the year and can cause episodes of diarrhea in adult animals (Braga et al., 2009). Anthelminthic drugs are usually used to control these gastrointestinal parasites. However, Bird and Herd (1995) report that no drug has efficacy against encysted larval stages of these nematodes.

Biological control with predatory nematophagous fungi, especially *Duddingtonia flagrans* are considered as a viable alternative (Braga et al., 2009). One of the principal advantages is the ability to survive for long periods under laboratory conditions; however, some isolates may lose their predatory activity (Stirling, 1991).

In this context, the use of silica-gel allows the storage of isolated spores for prolonged periods. According to Ryan et al. (2000) this method has been considered as one of the most effective, preserving the fungi for long periods. The main advantages are: (1) reduced chances contamination due to low humidity. (2) The financial cost is low and no specific equipment is required. (3) Many cultures of the same isolate may be recovered from a single sample storage. However, there is a lack of studies to assess whether the duration of preservation in silica-gel can interfere with the predatory activity.

The current study evaluates the predatory activity of the fungus *D. flagrans* (isolates AC001 and CG722) stored for 7 years in silica-gel. The fungal isolates predatory activity was tested against cyathostomin infective larvae (L_3).

2. Materials and methods

2.1. Fungi

Two isolates of nematophagous fungi *D. flagrans* (AC001 and CG722) were used. The isolates were stored in test tubes containing 2% corn-meal-agar (2% CMA), in the dark, at 4 °C for 10 days. These isolates were obtained from Brazilian agricultural soil, in Viçosa city, Zona da Mata region of Minas Gerais state. They were

* Corresponding author. Fax: +55 31 3899 1464.
E-mail address: fabioribeirobraga@hotmail.com (F.R. Braga).
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collected using the soil-sprinkling method of Duddington (1955), modified by Santos et al. (1991).

2.2. Maintenance of fungal cultures in vials containing silica-gel

Samples of the isolates AC001 and CG722 removed from test tubes containing 2% CMA, at 25 °C, were inoculated in sporulation medium AELA (yeast extract, 4 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H2O$, 0.5 g; soluble starch, 20 g; distilled water, 1 l) as described by Mota et al. (2002). After 7 days, a sterile solution of 5% skimmed milk and rice grains was added to the surface of the plates. Then the grains were soaked in the suspension of fungal material and transferred to five vials containing grains of sterile silica-gel. The samples were stored at 4 °C in the absence of light and humidity. After 7 years, the cultures were recovered by transferring a grain of rice containing the fungus *D. flagrans* (AC001 and CG722) from the bottle with silica-gel to the surface of 9.0 cm Petri dishes containing 20 ml of potato-dextrose agar 2% (2% PDA) and kept at 25 °C, in the dark, for 10 days.

2.3. Conidia collection

After growth of the isolates in 2% PDA, new culture disks 4 mm in diameter were transferred to 9.0 cm Petri dishes containing 20 ml of 2% water-agar (2% WA) and 1 ml of distilled water containing 1000 larvae of *Panagrellus* sp., were added daily for 21 days to induce fungal conidia formation. When complete fungal development was observed, 5 ml of distilled water were added to each Petri dish, and the conidial and mycelial fragments were removed using technique described by Araújo et al. (1993). The suspension present in the plates was screened through a sieve coupled to a plastic container to remove the mycelium fragments.

2.4. Obtaining cyathostomin infective larvae (L_3)

2.4.1. Fecal samples

About 1 kg of fresh feces were collected directly from the rectum of horses (*Equus caballus*) naturally infected with cyathostomins. These animals were from the Veterinary Department, Federal University of Viçosa, Minas Gerais, Brazil. After that, a count of eggs per gram of feces (EPG) was made according to Gordon and Whitlock (1939), in order to find positive animals. Then coprocultures using fragmented industrial vermiculite (autoclaved) and water were performed. The coprocultures were maintained at 26 °C in the dark for 8 days. At the end of this period cyathostomin (L_3) were obtained by the Baermann method, identified and quantified according to the criteria described by Bevilaqua et al. (1993) using an optical microscope with a 10× objective.

2.5. Experimental assay

The predation test was conducted on the surface of Petri dishes according to a modified technique described by Mota et al. (2002). Three groups were formed on 9.0 cm diameter Petri dishes containing 20 ml of 2% WA; two treated groups (AC001 and CG722) and one control group (without fungus). Six replicates were made for each group. Petri dishes were previously marked in 4 mm diameter fields. In the treated groups each Petri dish contained 500 cyathostomin L_3 and 500 conidia of isolates AC001 or CG722 in 2% WA. The control group contained only 500 L_3 on dishes in 2% WA. For 7 days, every 24 h, 10 random 4 mm diameter fields on each plate of treated and control groups were observed under an optical microscope with a 10× objective, counting the number of non-predated L_3 on each. At the end of 7 days, the non-predated L_3 were recovered from the Petri dishes using the Baermann apparatus with water at 42 °C.

2.6. Statistical analysis

Data obtained were examined by analysis of variance at 1% and 5% probability levels using the BioEstat 3.0 software (Ayres et al., 2003). The efficiency predation activity was evaluated using Tukey's test at 1% probability level. The percent reduction in the mean larval recovery was calculated by the following equation:

$$\text{Reduction}\% = \frac{(\text{Mean of } L_3 \text{ recovered from control} - \text{mean of } L_3 \text{ recovered from treatment}) \times 100/}{\text{Mean of } L_3 \text{ recovered from control}}$$

3. Results

Cultures of *D. flagrans* (isolates AC001 and CG722) preserved with silica-gel for 7 years were viable and produced conidia able to predate cyathostomin larvae. The presence of cyathostomin L_3 in Petri dishes containing 2% WA was essential for the trap formation by fungal isolates, since this medium is poor in nutrients.

A difference ($p < 0.01$) was observed between the means number of non-predated cyathostomin L_3 and the means in the groups treated with the fungus *D. flagrans* (AC001 and CG722) throughout the experiment (Table 1). Evidence of predation was observed by means of cyathostomin L_3 recovered on the seventh day using the Baermann method, at the end of the experiment. In addition, there were typical fungal structures (conidia and traps) in the treated groups during the test (Fig. 1A–E). At the end of the experiment, a mean cyathostomin L_3 reduction of 88.6% (AC001) and 78.4% (CG722) was observed (Fig. 2).

4. Discussion

Eysker et al. (1989) mention that adults cyathostomin can be controlled by many different broad spectrum anthelminthics. However, there are some disadvantages related to the administration of these drugs including the lack of efficacy against cyathostomins during encasement and the emergence of benzimidazole

Table 1
Daily means and standard deviations of non-predated third stage larvae (L_3) of cyathostomins per 4 mm diameter field in 2% water-agar during 7 days in treatments with the fungus *Duddingtonia flagrans* (AC001 and CG722) and control without fungus.

Time (Days)	Treatments (means of non-predated L_3)		
	AC001	CG722	Control
1	$4.2^a \pm 2.5$	$5.2^a \pm 3.8$	$26.3^b \pm 18.7$
2	$2.7^a \pm 2.6$	$3.1^a \pm 3.5$	$14.1^b \pm 24.7$
3	$2.9^a \pm 2.7$	$3.9^a \pm 2.8$	$10.6^b \pm 10.2$
4	$2.0^a \pm 1.9$	$2.3^a \pm 2.9$	$6.5^b \pm 7.1$
5	$1.3^a \pm 1.5$	$2.1^a \pm 2.2$	$5.9^b \pm 5.4$
6	$1.6^a \pm 1.7$	$1.7^a \pm 2.1$	$4.7^b \pm 4.4$
7	$0.9^a \pm 1.2$	$0.8^a \pm 1.1$	$3.8^b \pm 3.6$

Means followed by the same small letter in the lines were not statistically different ($p > 0.01$).