Journal of Helminthology (2009) **83,** 303–308
doi:10.1017/S0022149X09232342

Predatory activity of the fungi *Duddingtonia flagrans*, *Monacrosporium thaumasium*, *Monacrosporium sinense* and *Arthrobotrys robusta* on *Angiostrongylus vasorum* first-stage larvae

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(Accepted 18 December 2008; First Published Online 16 February 2009)

Abstract

Angiostrongylus vasorum is a nematode that parasitizes domestic dogs and wild canids. We compared the predatory capacity of isolates from the predatory fungi *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34), *Monacrosporium sinense* (SF53) and *Arthrobotrys robusta* (I31) on first-stage larvae (L_1) of *A. vasorum* under laboratory conditions. L_1 *A. vasorum* were plated on 2% water-agar (WA) Petri dishes marked into 4 mm diameter fields with the four grown isolates and a control without fungus. Plates of treated groups contained each 1000 L_1 *A. vasorum* and 1000 conidia of the fungal isolates AC001, NF34, SF53 and I31 on 2% WA. Plates of the control group (without fungus) contained only 1000 L_1 *A. vasorum* on 2% WA. Ten random fields (4 mm diameter) were examined per plate of treated and control groups, every 24 h for 7 days. Nematophagous fungi were not observed in the control group during the experiment. There was no variation in the predatory capacity among the tested fungal isolates ($P > 0.05$) during the 7 days of the experiment. There was a significant reduction ($P < 0.05$) of 80.3%, 74.5%, 74.2% and 71.8% in the means of *A. vasorum* L_1 recovered from treatments with isolates AC001, NF34, SF53 and I31, respectively, compared to the control without fungi. In this study, the four isolates of predatory fungi were efficient in the *in vitro* capture and destruction of *A. vasorum* L_1, confirming previous work on the efficiency of nematophagous fungi in the control of nematode parasites of dogs and as a possible alternative method of biological control.

Introduction

Angiostrongylus vasorum (Baillet, 1866) Kamensky, 1905, is a protostrongylid nematode of cosmopolitan distribution. Adult parasites can be found in the right ventricle and pulmonary arteries and their branches, causing severe consequences to the definitive host. The most common symptoms are cough, breathlessness, exercise intolerance, weight loss, neurological signs, heart failure and death (Ribieri *et al.*, 2001; Oliveira *et al.*, 2005; Saeed *et al.*, 2006). Infection of the definitive host results from the ingestion of infected snails, although paratenic hosts, such as frogs, lizards, mice and rats, may also be ingested by the definitive host (Bolt *et al.*, 1993).

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Angiostrongylus vasorum has a heteroxenic life cycle and several species of terrestrial and aquatic molluscs serve as intermediate hosts. Egg embryonation occurs in the pulmonary arterioles of the definitive host until the development of the first-stage larvae (L_1) that will hatch and penetrate the alveoli and enter the bronchioles and the bronchi. These L_1 migrate to the trachea and are either expelled with pulmonary secretions or swallowed and eliminated with faeces (Patteson *et al.*, 1993; Cury & Lima, 1995, 2002; Cury *et al.*, 2002). The L_1 remain in the faeces or may reach water collection points. Terrestrial or aquatic molluscs are infected by the ingestion of L_1 via the digestive tract or by larval penetration through their soft parts (Thiengo, 1996). Within mollusc tissues, larvae develop and become infective or third-stage larvae (Guilhon & Cens, 1973; Barçante *et al.*, 2003a).

Because of the medical importance of angiostrongyliasis in humans and animals, studies have been carried out to determine the efficacy of treatment with albendazole (Eckert & Lämmler, 1972). Most anthelmintic drugs are not efficient against this parasite within the definitive host. For this reason, alternative measures that may be used to combat the environmental spread and the infective forms of these parasites are important; such is the case of natural antagonists. These organisms may include different types of fungi, characterized as predators, endoparasites and opportunists. The advantages of their use include: they are not nutritionally demanding, do not require complex growth media, and concentrate their action on the faecal environment and combat free-living larvae (Araújo *et al.*, 2004a).

Until 1964, most fungi were classified as belonging to the genera *Arthrobotrys*, *Dactylis*, *Dactyella* and *Trichothecium*. Subsequently, several new genera were described, including *Duddingtonia*, *Monacrosporium*, *Geniculate* and *Dactylariopsis* (Gray, 1987). The vast majority of nematophagous fungi are mitosporic and were formerly classified into the division Deuteromycetes, class Hyphomycetes, order Hyphomycetales and family Moliniaceae. Recently, stages of sexual reproduction of these fungi have been observed for some species that are recognized as belonging to the phylum Ascomycota (Griffin, 1994).

Predatory fungi are certainly the most studied groups and show the greatest potential for biological control (Nordbring-Hertz *et al.*, 2002; Araújo *et al.*, 2007). The species *Duddingtonia flagrans*, *Monacrosporium thaumasium*, *M. sinense* and *Arthrobotrys robusta* are identified as nematophagous fungi and have been studied for their potential as biological control agents for gastrointestinal nematodes of domestic animals (Araújo *et al.*, 2006, 2008; Campos *et al.*, 2007).

Duddingtonia flagrans is considered the most promising nematode-trapping species for the control of nematodiosis in domestic animals, due to its plentiful production of chlamydospores. These thick-walled spores caused significant larval reduction in faecal cultures and pastures (Rocha *et al.*, 2007). It has predatory action by forming traps characterized by a system of simple adhesive hyphae that produce two types of spores – conidia and chlamydospores – interspersed by mature hyphae, which can be used as a biological control agent in the environment (Braga *et al.*, 2007). Species of the genus *Monacrosporium* are characterized by producing a single conidium on each conidiophore and production of adhesive networks, forming septate and branched hyphae, while *M. sinense* produces chlamydospores (Mota *et al.*, 2003; Campos *et al.*, 2007). The species *A. robusta* presents erect conidiophores, sometimes branched, about 300 mm in length, having the extremity increased in size, usually carrying six ovoid-shaped conidia, hyaline, sectioned closely to the intermediate region, 18–27 mm long and 8–12 mm wide, and capable of producing chlamydospores and adhesive networks for preying on nematodes (Araújo *et al.*, 1998, 2000).

The objective of this work was to compare the *in vitro* predatory capacity of the fungal species *D. flagrans*, *M. thaumasium*, *M. sinense* and *A. robusta* on first-stage larvae of *A. vasorum*.

Material and methods

Fungi

Four nematophagous fungal isolates were used in the experiment: *D. flagrans* (AC001), *M. thaumasium* (NF34), *M. sinense* (SF53) and *A. robusta* (I31). The isolates were originally obtained from soil located in Viçosa, Minas Gerais, Brazil, 20°45′20″S; 42°52′40″W, 649 m altitude, using the soil sprinkling method of Duddington (1955), modified by Santos *et al.* (1991). Fungi were kept in test tubes containing 2% cornmeal agar (2% CMA), in the dark, at 4°C for 10 days.

Conidia collection

Culture discs (4 mm in diameter) were removed from the fungal isolates kept in test tubes containing 2% CMA and transferred to 9.0 cm Petri dishes containing 20 ml of 2% potato dextrose agar and kept at 25°C in the dark for 10 days. After growth, new culture discs (4 mm in diameter) were transferred to 9.0 cm diameter Petri dishes containing 20 ml of 2% water-agar (2% WA) and 1 ml of distilled water containing 1000 larvae of *Panagrellus* sp. was added daily for 21 days to induce conidia formation. When fungal development was complete, 5 ml of distilled water were added to each Petri dish, and the conidial and mycelial fragments were removed as described by Araújo *et al.* (1993).

L_1 Angiostrongylus vasorum

The strain used in the assays was originally isolated from faeces of two naturally infected dogs, from the city of Caratinga, Minas Gerais (Lima *et al.*, 1985). This strain has been maintained by successive passages in dogs. Faeces of infected dogs were collected and placed in a modified Baermann funnel for 12 h, for L_1 recovery (Barçante *et al.*, 2003a, b). After this period, the tube was removed and 200 g were centrifuged for 2 min. The supernatant was discarded and the pellet containing *A. vasorum* L_1 was resuspended in 5 ml of 0.85% saline solution. The content was homogenized and three 10 µl aliquots were removed and distributed on 7.5 × 2.5 cm glass slides. Larval counts were carried out under a stereomicroscope (25 ×). The total larval number was estimated by a simple rule of three.

https://doi.org/10.1017/S0022149X09232342

Experimental assay

Treatments consisted of four groups of fungal isolates and a control without fungus plated in 9.0 cm Petri dishes containing 20 ml of 2% WA, with six repetitions each. Petri dishes were previously marked into 4 mm diameter fields. A total of 1000 *A. vasorum* L_1 larvae were plated with 1000 conidia of one of the fungal isolates AC001, NF34, SF53 or I31 in 2% WA. The control group (without fungus) contained 1000 L_1 larvae plated with 2% WA only.

Ten random fields (4 mm diameter) were examined per plate of treated and control groups, every 24 h for 7 days under an optical microscope (10 × and 40 × objective lens) for non-predated L_1 counts. After 7 days, the non-predated L_1 were recovered from the Petri dishes using the Baermann method.

Statistical analysis

Means of recovered *A. vasorum* L_1 were calculated. Data were examined by analysis of variance at significance levels of 1 and 5% probability (Ayres *et al.*, 2003). Predation efficiency of L_1 relative to the control group was evaluated by the Tukey's test at 1% probability. The reduction percentage of L_1 means was calculated according to the following equation: Reduction % = (Average of L_1 recovered from control group − Average of L_1 recovered from treatment groups) × 100/Average of L_1 recovered from the control group.

Results

The tested isolates of predatory fungi *D. flagrans* (AC001), *M. thaumasium* (NF34), *M. sinense* (SF53) and *A. robusta* (I31) were able to capture *A. vasorum* L_1 in the experimental *in vitro* test.

No difference was found ($P > 0.05$) in the comparison between capture and destruction of *A. vasorum* L_1 in Petri dishes of the groups treated with isolates of *D. flagrans* (AC001), *M. thaumasium* (NF34), *M. sinense* (SF53) and *A. robusta* (I31) during the experimental assay (table 1), but there was difference ($P < 0.05$) between the means of non-predated *A. vasorum* L_1 per 4 mm diameter field in the Petri dishes of the control group compared to means of L_1 recorded in the groups treated with the fungi throughout the experiment. The recorded reduction percentages of *A. vasorum* L_1 were 80.3% (AC001) 74.5% (NF34), 74.2% (SF53) and 71.8% (I31).

The presence of *A. vasorum* L_1 in Petri dishes containing 2% WA was essential for trap formation by the fungal isolates, since this culture medium is nutritionally poor (fig. 1A and B). The presence of nematophagous fungi was not observed in the control group during the experiment. Evidence of predation was confirmed by the means of recovered *A. vasorum* L_1 using the Baermann method at 7 days post-plating, at the end of the experiment (fig. 2).

The linear regression coefficients calculated by the analysis of *A. vasorum* L_1 means per 4 mm diameter field of the treated and control groups were: −0.075 (AC001); −0.190 (NF34); −0.311 (SF53); −0.256 (I31) and −0.282 (control group).

Discussion

The genera *Ancylostoma* and *Toxocara*, parasites of dogs and cats, and possibly the species *Angiostrongylus vasorum*, stand out among helminths with zoonotic potential. Besides showing medical–veterinary importance, *A. vasorum* as a cardiopulmonary parasite of domestic and wild dogs also requires special attention and investigation because it can also infect humans (Eckert & Lämmler, 1972; Lima *et al.*, 1994).

Nematophagous fungi, especially the genera *Duddingtonia*, *Monacrosporium* and *Arthrobotrys*, have predatory capacity on infective larvae (L_3) of gastrointestinal nematode parasites of domestic animals (Araújo *et al.*, 2004b, 2006; Campos *et al.*, 2007). These genera are recognized only as predators, and the predatory capacity of the species *D. flagrans* and *M. thaumasium*, *M. sinense* and *A. robusta* has been thoroughly discussed and proved in the control of L_3 of gastrointestinal nematode parasites of ruminants, horses and small ruminants (Castro *et al.*, 2003; Chandrawathani *et al.*, 2004; Araújo *et al.*, 2006, 2007; Dias *et al.*, 2007). However, the predatory capacity of these species had never been tested on larvae of *A. vasorum*. This is the first work on the interaction of the fungi *D. flagrans*, *M. thaumasium*, *M. sinense* and *A. robusta* with *A. vasorum* L_1 larvae. Few studies have mentioned the *in vitro* predatory activity of different nematophagous

Table 1. Daily means and standard deviations of non-predated first-stage larvae (L_1) of *Angiostrongylus vasorum* per 4 mm diameter field in 2% water-agar during 7 days of treatment with the fungal isolates *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34), *Monacrosporium sinense* (SF53), *Arthrobotrys robusta* (I31) and control without fungus.

	Treatment				
Time (days)	AC001	NF34	SF53	I31	Control
1	0.98 ± 2.04a	1.23 ± 3.07a	2.10 ± 3.50a	2.73 ± 4.22a	14.73b ± 6.81
2	2.59 ± 4.12a	4.34 ± 4.99a	2.32 ± 3.32a	1.80 ± 3.29a	23.51b ± 13.02
3	0.40 ± 0.78a	1.15 ± 1.51a	0.58 ± 0.96a	0.93 ± 1.05a	14.20b ± 6.31
4	0.68 ± 1.62a	1.02 ± 1.94a	0.54 ± 1.16a	1.02 ± 1.72a	12.10b ± 6.66
5	0.41 ± 1.24a	1.05 ± 1.26a	0.39 ± 0.70a	0.98 ± 1.37a	12.90b ± 9.22
6	0.88 ± 1.99a	1.51 ± 2.54a	0.54 ± 1.53a	0.66 ± 2.02a	11.39b ± 6.74
7	1.33 ± 2.70a	1.38 ± 2.65a	0.45 ± 0.68a	1.08 ± 1.87a	11.28b ± 8.71

Means followed by the same small letter in the rows were not statistically different ($P > 0.05$).

https://doi.org/10.1017/S0022149X09232342

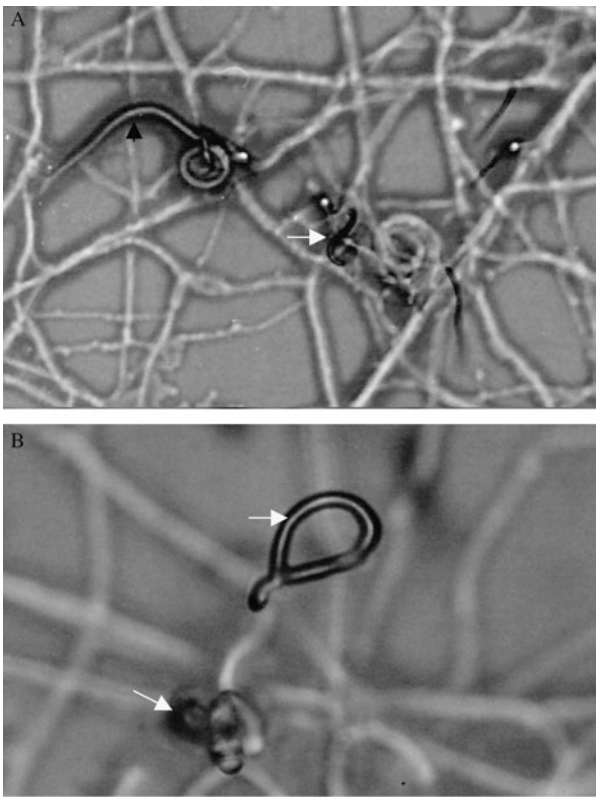

Fig. 1. *Angiostrongylus vasorum* first-stage larvae (L_1) captured by *Duddingtonia flagrans* (black arrow) in Petri dishes containing 2% water-agar, and trap formation by the fungal isolate *D. flagrans* (white arrow). Magnification: (A) 10 × and (B) 40 × objective lens.

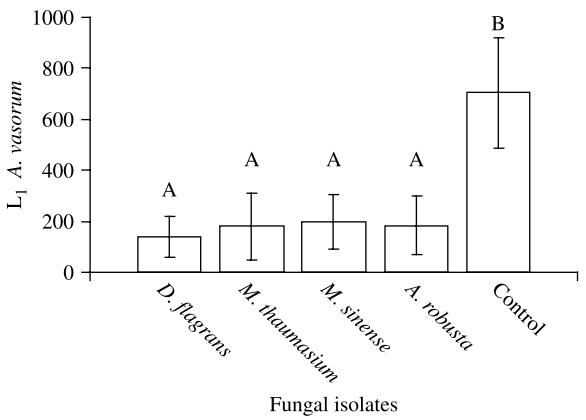

Fig. 2. Mean number of non-predated *Angiostrongylus vasorum* first-stage larvae (L_1) recovered in 2% water-agar by the Baermann method on the seventh treatment day after interaction with the fungal isolates *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34), *Monacrosporium sinense* (SF53), *Arthrobotrys robusta* (I31) and control (without fungus). Lines on bars represent standard deviation. Means followed by at least one common capital letter (A) in the row are not significantly different by Tukey's test at a 5% probability level.

fungi on larvae of nematode parasites of dogs (Maciel *et al.*, 2006a, b).

In the present work, although the isolate (I31) from *A. robusta* was effective in reducing *A. vasorum* L_1 with reduction percentage of 71.8%, *D. flagrans* (AC001) was found to be more efficient compared with the others and at the end of 7 days showed a higher reduction percentage (80.31%). These results showed that *D. flagrans* (AC001) had higher *in vitro* predatory activity on *A. vasorum* L_1.

Eren & Pramer (1965) reported on the regular supply of nematodes to fungi in nutritionally poor culture media, which reduces fungal saprophytic growth. For this reason, in this study we used only 2% WA, a low nutrient culture medium, to reduce the saprophytic growth of fungal isolates, providing *A. vasorum* L_1 as a single nutritional source. The higher the mobility of nematodes, the greater the stimulus to trap production (Nansen *et al.*, 1988). This phenomenon was observed in this study, as trap formation and L_1 predation by the fungal isolates were recorded during the first observation, 24 h after interaction (fig. 1). Maciel *et al.* (2006b) found that in 2% WA, the isolate *A. robusta* (I31) showed higher predatory activity on *Ancylostoma* spp. L_3 compared with isolates *D. flagrans* (CG768) and *M. thaumasium* (NF34). However, in the present study, the isolate *D. flagrans* (AC001) in 2% WA showed the highest *in vitro* predatory activity on *A. vasorum* L_1 at the end of the experiment.

Negative coefficients of correlation indicate a downward behaviour of the regression curves for the treatments with the fungal isolates AC001, NF34, SF53 and I31. This was caused by the reduction in the means of non-predated *A. vasorum* L_1 per 4 mm diameter field during the experimental assay, mainly by the capture of L_1 in fungal traps. Reduction in the number of L_1 per 4 mm diameter field in the control group, during the study, was caused by larval migration to the periphery of the Petri dishes, where the moisture level was higher, which was also reported by Araújo *et al.* (2006) in an *in vitro* assay carried out in Petri dishes.

The results of this study confirm previous work on the efficiency of nematophagous fungi in the control of larvae of nematode parasites of dogs. This allows us to conclude that the fungi *D. flagrans*, *M. thaumasium*, *M. sinense* and *A. robusta* have predatory activity on first-stage larvae of *A. vasorum* and that this is a likely alternative method for biological control of *A. vasorum* larvae.

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https://doi.org/10.1017/S0022149X09232342