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Short communication

First report of the activity of predatory fungi on *Angiostrongylus cantonensis* (Nematoda: Angiostrongylidae) first-stage larvae

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ABSTRACT

The nematode *Angiostrongylus cantonensis* causes eosinophilic meningoencephalitis in humans and thus alternative methods of control should be studied. The objective of this work was to evaluate the predatory capacity of eight fungal isolates of the species *Duddingtonia flagrans* (AC001, CG768 and CG722), *Monacrosporium thaumasium* (NF34), *M. sinense* (SF53) and *Arthrobotrys robusta* (I31), *A. cladodes* (CG719) and *A. conoides* (I40) on first-stage larvae (L_1) of *A. cantonensis* under laboratory conditions. The treated groups contained 1000 conidia of the fungal isolates and 1000 *A. cantonensis* L_1 in Petri dishes containing 2% water-agar medium (2% WA). The control group (without fungi) contained only 1000 *A. cantonensis* L_1 in 2% WA. Evidence of predation was observed at the end of 7 days. Percentage reductions in L_1 were: AC001, 82.8%; CG768, 71.0%; CG722, 72.8%; NF34, 86.7%; SF53, 89.7%; I40, 48.3%; CG719, 84.7%; and I31, 80.4%. No significant difference was observed ($p > 0.01$) between the actions of the isolates used; however, a difference was noted ($p < 0.01$) in relation to the control group. The results of the present work, confirm previous reports of the effectiveness of the fungi *D. flagrans*, *M. thaumasium*, *M. sinense* and *A. robusta* in controlling larvae of potentially zoonotic nematodes, this being the first report on *A. cantonensis* L_1.

1. Introduction

Angiostrongylus cantonensis (Chen, 1935) is a potentially zoonotic nematode and considered to be the main cause of human eosinophilic meningoencephalitis, occurring mainly in Asia, Pacific Islands, North America and South America (Alicata, 1962; Koo et al., 1988; Tsai et al., 2001; Diaz, 2008; Moreira et al., 2013).

In relation to its life cycle, rodents (*Rattus norvegicus* and *Rattus rattus*) have been reported as the definitive hosts of *A. cantonensis* (Yousif and Ibrahim, 1978; Foronda et al., 2010; Taylor et al., 2010). The larvae (first-stage; L_1) are released from the faeces of rodents into the environment and can be ingested by mollusks of the genera *Agrolimax*, *Limax* and *Deroceras*, and can even penetrate the tissue of these, which act as intermediate hosts. In these hosts, the larvae (L_1) will pass through two more changes until they reach their infective stage (third-stage; L_3) (Taylor et al., 2010). The life cycle is completed when the rats ingest slugs infected with L_3. Man acquires the infection after ingestion of the intermediate or paratenic host and through ingestion of rainwater in the field where this nematode is present (Wang et al., 2007).

In this context, the use of nematophagous fungi of the genera *Duddingtonia*, *Monacrosporium* and *Arthrobotrys* has been successfully tested, including on the *Angiostrongylus* genus, specifically on the *A. vasorum* species (Braga et al., 2009, 2011; Soares et al., 2012).

The fungus *Duddingtonia flagrans* is the most studied species in the control of gastrointestinal helminths from domestic animals. This fungus captures nematodes through adhesive hyphae. The species of the genus *Monacrosporium* are characterized by producing only one conidia on the end of a conidiophore and by capturing nematodes through adhesive networks. The genus *Arthrobotrys* includes a large number of species of nematophagous fungi and captures nematodes through adhesive networks (Araújo et al., 2004).

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The objective of this work was to evaluate the *in vitro* predatory capacity of eight fungal isolates of the genera *Duddingtonia*, *Monacrosporium* and *Arthrobotrys* on L_1 of *A. cantonensis*. This is the first report of the predatory activity of these fungi on the *A. cantonensis* species.

2. Materials and methods

2.1. Fungi and obtaining of the conidia

The species *D. flagrans* (AC001, CG768 and CG722), *Monacrosporium thaumasium* (NF34), *M. sinense* (SF53) and *Arthrobotrys robusta* (I31), *A. cladodes* (CG719) and *A. conoides* (I40) were utilized. These isolates are from soil in Brazil, in the town of Viçosa and have been kept in the Veterinary Department, Federal University of Viçosa. Nematophagous fungi produce traps and vegetative structures (conidia and/or chlamydospores) in the presence of nematodes, which are routinely used in the laboratory for this purpose (Braga et al., 2012). Thus, culture plates of 4 mm in diameter were extracted from fungal isolates maintained in test tubes containing 2% corn-meal-agar and transferred to Petri dishes of 9.0 cm in diameter containing 20 ml of 2% potato dextrose-agar kept at 25 °C in the dark for 10 days.

After growth of the isolates new culture plates of 4 mm in diameter were transferred to Petri dishes of 9.0 cm diameter containing 20 ml of 2% water–agar (2% WA). Daily, 1 ml of distilled water containing 1000 larvae *Panagrellus redivivus* was added during a period of 21 days to induce formation of conidia. When the complete fungal development was observed, 5 ml of distilled water were added to each Petri dish, and the mycelial fragments and conidia were removed according to the technique described by Araújo et al. (1993).

2.2. Obtaining of the Angiostrongylus cantonensis larvae

Larvae of *A. cantonensis* were obtained from naturally infected specimens of *Achatina fulica*, from the municipality of São Gonçalo, Rio de Janeiro, Brazil (Maldonado et al., 2010). The L_1 were recovered using the technique of artificial digestion (Graeff-Teixeira and Moreira, 1995) and inoculated by gavage in *R. norvegicus* and using *Biomphalaria glabrata* as an intermediate host in the Instituto Oswaldo Cruz (Fiocruz, RJ, Brazil), where the cycle has been maintained with the permission of the ethics committee for animal use (LW-24/10). The faeces of the parasitized *R. norvegicus* were collected to obtain larvae by the method of Baermann (1917) modified by Moraes (1948). The pellet was collected and centrifuged (2000 RPMI for 10 min) and analysed using a light microscope for the detection and recovery of L_1.

2.3. Experimental assay

A total of nine groups were formed in Petri dishes of 4.5 cm in diameter containing 10 ml of 2% WA: eight treatment groups and a control group, with six replicates for each group. The Petri dishes were previously marked in fields of 4 mm in diameter. In the treated groups each Petri dish contained 1000 L_1 of *A. cantonensis* and 1000 conidia of the fungal isolates (AC001, CG722, CG768, NF34, SF53, I40, CG719 and I31) in 2% WA, and the control group (without fungi) contained only 1000 L_1 in the dishes with 2% WA.

For 7 days, every 24 h, 10 random fields of 4 mm in diameter on each plate of the treated and control groups were observed under light microscopy at 10× objective, and the number of non-predated L_1 were counted in each one. After 7 days, the non-predated L_1 in the content of the Petri dishes were recovered through the Baermann apparatus with water at 42 °C.

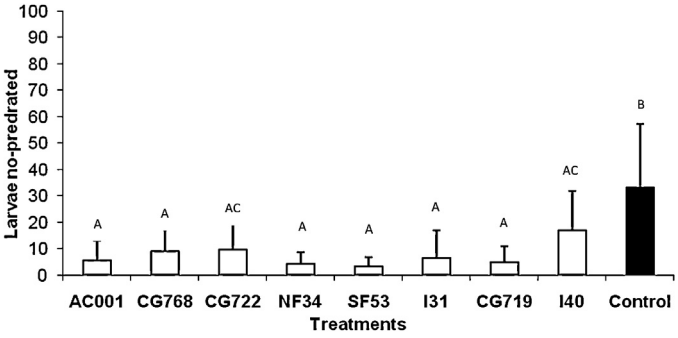

Fig. 1. Mean number of non-predated first stage larvae (L_1) of *Angiostrongylus cantonensis* recovered in medium 2% water–agar on the seventh day after the treatments with the different isolates of the fungi *Duddingtonia flagrans* (AC001, CG768 and CG722), *Monacrosporium thaumasium* (NF34), *M. sinense* (SF53), *Arthrobotrys robusta* (I31), *A. cladodes* (CG719) and *A. conoides* (I40) and of control (without fungi). Bar represents the standard deviation. Means followed by the same letter (A, B and C) in the lines were not statistically different ($P > 0.05$).

2.4. Statistical analysis

The average number of *A. cantonensis* L_1 recovered was calculated. The data were interpreted by analysis of variance with significance levels of 1% and 5% probability (Ayres et al., 2003). The efficiency of predation on L_1 compared with control was assessed by the Tukey test at 1% and 5% probability. Thereafter, the percentage of reduction from the average of L_1 was calculated according to the following formula:

$$\%\,\text{Reduction} = \frac{(\text{average of } L_1 \text{ recovered from control} - \text{average of } L_1 \text{ recovered from treatment})}{\text{Average of } L_1 \text{ recovered from control}} \times 100.$$

3. Results

The isolates of the predatory fungi tested, *D. flagrans* (AC001, CG722 and CG768), *M. thaumasium* (NF34), *M. sinense* (SF53), and *A. conoides* (I40), *A. cladodes* (CG719) and *A. robusta* (I31), were able to prey on the *A. cantonensis* L_1 in the *in vitro* experimental assay. Evidence of predation was observed at the end of the experiment (7 days), where the following percentages of reduction of non-predated L_1 were observed: AC001, 82.8%; CG768, 71.0%; CG722, 72.8%; NF34, 86.7%; SF53, 89.7%; I40, 48.3%; CG719, 84.7%; and I31, 80.4% (Fig. 1). It was observed no significant difference ($p > 0.01$) between the actions of isolates used at the end of 7 days; however, a difference was noted ($p < 0.01$) compared with the control group. On the other hand, it was observed that the action of the CG722 isolate was statistically similar to the action of the I40 isolate.

Every 24 h, predation and the formation of traps were visualized in all plates of the groups treated with the different genera of fungi used and control (Fig. 2a–d). However neither the presence nor formation of traps of nematophagous fungi in the control group was noticed.

Regarding daily predation, the results for the activity of the eight fungal isolates tested are shown in Table 1. In this particular case, there were significant differences ($p < 0.01$) between the groups treated with the fungi and the control group on days 1, 2, 5, 6 and 7. However, in the interval of the third day, it was observed that the isolates CG722 and CG719 showed no difference ($p > 0.01$) compared with the control group. Moreover, in the interval of the fourth day the isolates AC001, CG768, CG722, NF34, I31 and I40 showed no difference ($p > 0.01$) compared with the control group.

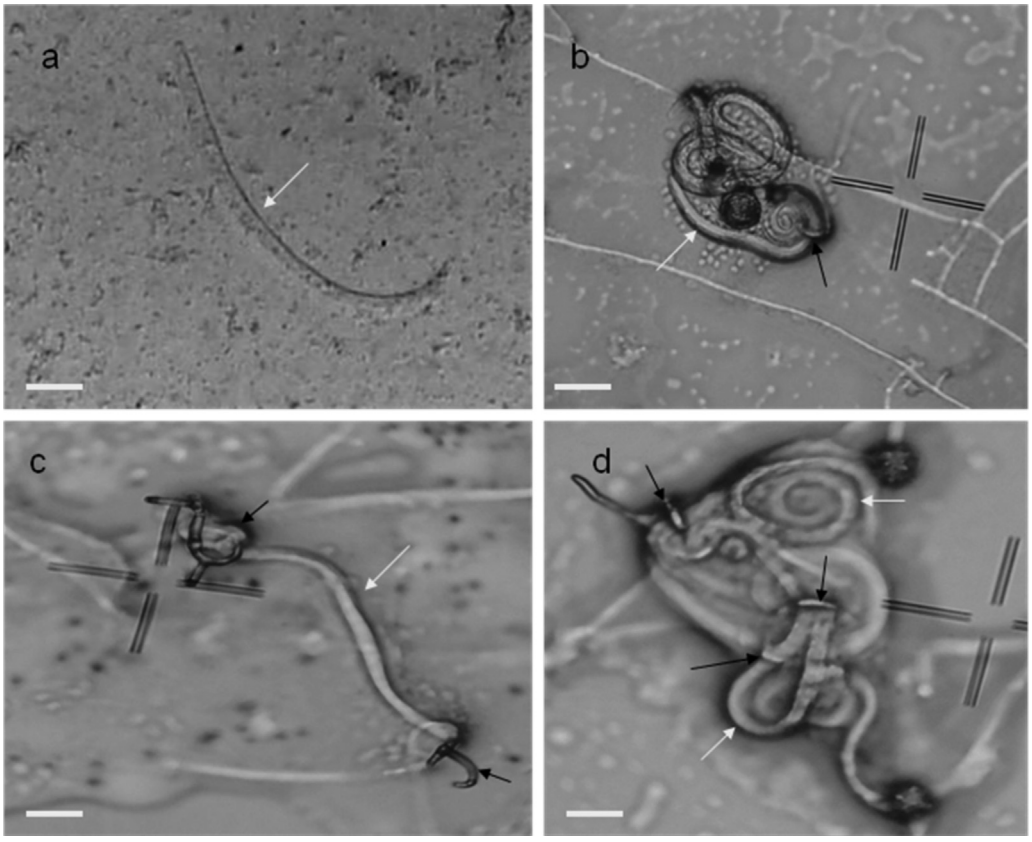

Fig. 2. (a) Control (white arrow), (b) trap formation (black arrow) by the tested fungal isolates *Duddingtonia*, (c) *Monacrosporium*, (d) *Arthrobotrys* and predated first stage larval of *A. cantonensis* (white arrow). Optical microscopy – 40× objective lens. Bars: (A) 14 μm; (B) 4.2 μm; (C) 14.2 μm and (D) 7.2 μm.

Table 1
Daily mean and standard deviations of non-predated first stage larvae of *Angiostrongylus cantonensis* during the period of seven days in treatments with the fungi *Duddingtonia flagrans* (AC001, CG768 and CG722), *Monacrosporium thaumasium* (NF34), *M. sinense* (SF53), *Arthrobotrys robusta* (I31), *A. cladodes* (CG719) and *A. conoides* (I40) and in control under Petri dishes of 4.5 cm in diameter containing 10 ml of 2% water–agar (2% WA).

Fungi	Time (Days)						
	1	2	3	4	5	6	7
AC001	$7.83^{A} \pm 7$	$8.8^{A} \pm 10$	$4.2^{A} \pm 6$	$3.5^{A} \pm 4$	$2.8^{A} \pm 3$	$2.8^{A} \pm 4$	$2.3^{A} \pm 4$
CG768	$14.5^{BA} \pm 10$	$6.9^{A} \pm 5$	$4.0^{AB} \pm 3$	$5.5^{A} \pm 3$	$6.3^{A} \pm 7$	$6.1^{A} \pm 10$	$2.7^{A} \pm 7$
CG722	$14^{BA} \pm 11$	$16.9^{BB} \pm 15$	$20.6^{BA} \pm 22$	$11.3^{B} \pm 13$	$11.0^{BA} \pm 7$	$14.1^{B} \pm 14$	$11.5^{BA} \pm 16$
NF34	$14.0^{BAA} \pm 8$	$7.7^{AAB} \pm 9$	$5.2^{AAB} \pm 5$	$4.6^{AA} \pm 3$	$2.2^{AB} \pm 2$	$2.7^{A} \pm 3$	$4.0^{AB} \pm 4$
SF53	$11.0^{AAAA} \pm 12$	$4.5^{AABA} \pm 3$	$4.2^{AABA} \pm 4$	$2.7^{AA} \pm 2$	$2.3^{AB} \pm 1$	$1.6^{A} \pm 1$	$1.5^{AB} \pm 1$
131	$6.3^{ABBBA} \pm 5$	$11.5^{AAAAB} \pm 5$	$4.4^{ABBAA} \pm 9$	$9.2^{BABAB} \pm 11$	$5.7^{AB} \pm 4$	$7.7^{AB} \pm 12$	$6.4^{AB} \pm 11$
CG719	$9.0^{AAAAAAA} \pm 7$	$16.4^{BBABBA} \pm 10$	$13.9^{BBBBBA} \pm 11$	$14.7^{BBBABA} \pm 11$	$11.8^{BA} \pm 10$	$8.2^{BAB} \pm 7$	$9.7^{BAB} \pm 9$
I40	$10.0^{AAAAAAA} \pm 7$	$17.7^{BBABBBA} \pm 12$	$12.0^{BBBBBAA} \pm 13$	$10.8^{BBBABA} \pm 12$	$10.3^{BABA} \pm 9$	$7.4^{AB} \pm 8$	$6.0^{AB} \pm 9$
Control	$30.8^{C} \pm 16$	$24.^{C} \pm 16$	$18.3^{CBAB} \pm 12$	$8.2^{AABABA} \pm 8$	$63.8^{C} \pm 22$	$34.1^{C} \pm 11$	$27.2^{C} \pm 28$

Means followed by the same letter (A, B and C) in the columns were not statistically different ($P > 0.01$).

4. Discussion

D. flagrans (AC001) showed at the end of 7 days a reduction of 80.3% (Braga et al., 2009) in comparison with 82.8% (AC001) found in this study on *A. cantonensis*. However, there are no reports of the use of isolated CG768 on *A. cantonensis*, and so comparisons with other potentially zoonotic helminth larvae should be performed. Thus, the activity of this isolate was tested on *Ancylostoma* spp. L_3 with a percentage reduction of 92.8% (Maciel et al., 2009), this result is consistent with our report where it was observed a percentage reduction of 72.8%, demonstrating its effectiveness even on another helminth L_3. Finally, for the isolate CG722, in this work was found a percentage reduction in L_1 *A. cantonensis* of 70.9%; however, there are no reports of its predation on larvae of potentially zoonotic helminths.

Following this line of reasoning, for *Monacrosporium* genus, the species *M. thaumasium* (NF34) and *M. sinense* (SF53) were successfully tested on *A. vasorum* L_1 by Braga et al. (2009). NF34 and SF53 reduced *A. vasorum* L_1 by 74.5% and 74.2%, respectively, compared with 86.8% and 89.7% reductions in *A. cantonensis* L_1 in the present work.

For the genus *Arthrobotrys*, the species *A. robusta* (I31) has proved to be effective on *A. vasorum* L_1, showing a reduction of 71.8% (Braga et al., 2009) compared with the present study (80.4%). The species *A. cladodes* (CG719) and *A. conoides* (I40) were tested on *Ancylostoma* spp. L_3 *in vitro*; at the end of the experiment percentage reductions of 71.3% and 76.9% were obtained (Carvalho et al., 2009), whereas the percentages obtained were 89.7% and 48.3%, respectively. On the other hand, reduction in the number of L_1 per 4 mm diameter field in the control group, during the work,

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.

In the interval of the fourth day the isolates AC001, CG768, CG722, NF34, I31 and I40 showed no difference ($p > 0.01$) compared with the control group. This strongly suggests that the motility of *A. cantonensis* L_1 was not fully effective; in this case the low production of traps and consequently the reduced efficacy of these fungi, in this particular 2 days, may be explained. Evidence of predatory activity of all fungal isolates tested could be observed at the end of the experiment through the recovery of non-predated L_1. These results are in agreement with Gomes et al. (1998) who observed no significant reductions in infective larvae (L_3) of *Oesophagostomum* sp. of cattle in the pasture for this genus.

In these work, the isolates used belonged to the genus *Arthrobotrys* and variations in predatory behaviour of isolates from different species are common (Araújo et al., 1993), which could explain the results found for the isolate of *M. sinense*.

This work is an important step in understanding the interaction of fungi *versus A. cantonensis* L_1, even as an experimental design. In this context, the applicability of nematophagous fungi is suggested, highlighting the use of any tested isolate (AC001, CG768 and CG722) from *Duddingtonia* genus (NF34 and SF53), *Monacrosporium* genus (I31, CG719) and *Arthrobotrys* genus that is able to destroy *A. cantonensis* L_1 in laboratory conditions. However, perfect conditions on the use of these fungi in controlling *A. cantonensis* L_1 must still be studied exhaustively.

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