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In-Vitro Sensitivity of Infective Larvae of Oesophagostomum Species to Nematophagous Fungi

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Two experimental assays (A and B) evaluated the action of conidia of the nematophagous fungi Duddingtonia flagrans (AC001), Monacrosporium sinense (SF53) and Artrhobotrys robusta (I-31) against infective larvae (L3) of Oesophagostomum spp in 2% water-agar (2%WA) medium and coprocultures. The first assay consisted of three groups of 1000 Oesophagostomum L3 treated with 1000 conidia of isolates AC001, SF53 and I-31 and control without fungus plated in 2% WA. In the second assay, 1000 conidia of the same isolates were added to 20g of feces and incubated at 26°C for 8 days. The L3 no predate were recovered after this period fungal treatment. There was no variation in the predatory capacity among the tested fungal isolates (p>0.01) during the experimental period of seven days. There were significant reductions (p<0.01) of 94.4%, 92.9% , and 88.3% in the means of Oesophagostomum L3 recovered from the treatments with isolates AC001, SF53 and I-31, respectively, when compared to the control treatment. Second assay also showed statistical differences (p<0.01) between the means of recovered L3 in the treated groups and the control, with the following percent reductions: 75.3% (AC001), 63.7% (SF53) 63.3% (I-31). In this study, the three isolates of predatory fungi D. flagrans (AC001), M. sinense (SF53) and A. robusta (I-31) were efficient in the in vitro capture and destruction of Oesophagostomum L3 and are potential biological control agents of this nematode.

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INTRODUCTION

Helminths that infect pigs vary widely in size, life cycle and pathogenicity. Diseases caused by internal and external parasites have been reported in pig farms worldwide. The infections are not always apparent and persist at subclinical levels for extended periods, leading even to death of animals [1]. The genus Oesophagostomum, with worldwide distribution, is among the main intestinal helminths infecting pigs. The major species are *O. dentatum* and *O. quadrispinulatum* [2]. Oesophagostomum infection occurs through the ingestion of infective larvae (L_3) present in the environment. Roepstorff and Jorsal [3] reported the occurrence of helminths in 66 pig herds in Denmark of which 58% were Oesophagostomum spp. infestations.

Roepstorff and Nansen [4] pointed out that the expanding technology in production systems improves sanitary conditions by creating monitoring systems that reduce chances of parasite transmission. However, pigs raised extensively on pasture demand reformulated control measures against parasites, with special attention to the control of free-living infective stages. In this case, the control with nematophagous fungi is suggested because their action is concentrated in the fecal environment and directed against the free-living forms [5].

Nematophagous fungi are classified into predators, opportunists and endoparasites. The genera Duddingtonia, Monacrosporium and

Arthrobotrys of the predator group are the most studied for the control of nematodiosis in domestic animals [6]. *D. flagrans* is considered the most promising because of the production of large amounts of chlamydospores. *M. thaumasium* and *A. robusta* have been successfully used in the control of infective larvae of gastrointestinal nematodes of domestic animals in laboratory and field conditions [7, 8, 9]. However, there are not reports on the action of nematophagous fungi in the control of gastrointestinal nematodes of pigs.

This study evaluated the in vitro predatory activity of the fungi *D. flagrans*, *M. sinense* and *A. robusta* against larvae of Oesophagostomum spp in two experimental assays.

MATERIAL AND METHODS

1) Fungal culture

Isolates of *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) were obtained from soil in Viçosa, Zona da Mata region, State of Minas Gerais, between 20°45'20" S and 42°52'40" W longitude, 649 m altitude. The isolates were kept in test tubes with 2% corn-meal-agar (2% CMA), at 4°C in the Parasitology laboratory of the Federal Universidad of Viçosa.

2) Conidia Collection

Culture disks (4 mm in diameter) of fungal isolates in 2% CMA were removed from the test tubes 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 disks (4 mm in diameter) were transferred to 9.0 cm diameter Petri dishes containing 20 ml of 2% water-agar (2% WA). Distilled water (1 ml) containing 1000 larvae of Panagrellus sp was daily added to the plates to induce conidial formation for 21 days. After complete fungal development, 5 mL of distilled water were added to each plate and conidia and mycelial fragments were removed using technique described by Carvalho et al. [10].

3) Oesophagostomum spp L_3

Infective larvae (L_3) of Oesophagostomum spp. were recovered from feces of naturally infected pigs using vermiculite coproculture for 15 days. At the end of this period, third stage larvae (L_3) were obtained by the Baermann method, with water at 42-45°C and 12h decantation time. Larvae were identified as per Ueno and Gonçalves [11].

4) Assays

Two in vitro assays (A and B) were carried out with an interval of eight days. Assay A evaluated fungal activity of conidia of *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) on OesophagostomumL_3 in 2% WA.

Assay B evaluated the action of conidia of isolates AC001, SF53 and I-31 in coprocultures containing Oesophagostomum larvae.

5) Assay A

Assay A consisted of four treatments of fungal isolates (AC001, SF53 and I-31) and a control without fungus plated in 9.0cm Petri dishes containing 20 ml of 2%WA, with six repetitions each. Petri dishes were marked into 4mm fields. A thousand Oesophagostomum L_3 were plated with 1000 conidia of the fungal isolates. The control contained 1000 L_3 without fungus, according to the methodology used by Braga et al. (2010a). Ten random fields (4mm diameter) were examined per plate of the treated and control groups, using an optical microscope (10 X objectives) for L_3 counts, every 24 hours, for seven days. After 7 days, the non-predated L_3 were recovered from the Petri dishes using the Baermann method, with water at 42 ºC [12].

6) Assay B

Fresh feces with positive EPG were used for preparing coprocultures, which were mixed with autoclaved and moistened vermiculite. Treatments consisted of four groups of fungal isolates (AC001, SF53 and I-31) and a control without fungus, with six repetitions each. Each replicate received 1000 fungal conidia. The control group contained fecal culture only. Coprocultures, from treated and control groups, were incubated at 26°C and for 8 days. At the end of this period, L_3 were recovered by the Baermann method, which were identified and quantified according to the criteria described by Ueno and Gonçalves [11] under optical microscope (10 x objectives).

7) Statistical analysis

Means of Oesophagostomum L_3 recovered from tests A and B were examined by analysis of variance at 1% probability level [13]. Predation efficiency of L_3 relative to the control group was assessed by the Tukey's test at 1% probability level. The percent reduction in means of recovered L_3 was calculated by the following equation:

Reduction % = [(Mean of L_3 recovered from control group - Mean of L_3 recovered from treated group) /Mean L_3 recovered from control group]x100

RESULTS

Isolates of predatory fungi *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) were capable to prey Oesophagostomum L_3 in assay A.

No significant difference ($p>0.01$) was found in the comparison of capture and destruction of Oesophagostomum L_3 among plates of the groups treated with isolates AC001, SF53 and I-31 during the experimental assay (Table 1). However, there was difference ($p<0.01$) between the means of non-predated Oesophagostomum L_3 per 4mm diameter field in the plates of the control group and means of L_3 recorded in the fungi-treated groups. The recorded percent reduction in Oesophagostomum L_3 was: 94.4% (AC001), 92.9% (SF53) and 88.3% (I-31).

Nematophagous fungi were not observed in plates of the control group during the experiment, so this shown variation in the percentage of larvae observed in the table, probably, due to migration of larvae to regions of the plates where there was more humidity. The presence of Oesophagostomum L_3 was essential for trap formation in plates containing nutrient-poor medium such as 2% WA. Evidence of predation was confirmed by the means of recovered L3 using the Baermann method 7 days post-plating, at the end of the assay A (Fig. 1). Difference ($p<0.01$) was found between the number of recovered L_3 in the treated groups and the control without fungi.

At the end of 8 days, means of recovered larvae in assay B showed that fungal conidia of *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) were effective in reducing Oesophagostomum L_3 (Fig. 2). Significant difference ($p<0.01$) was found between the means of larvae recovered from the treated groups and the control group with the following percent reductions: 75.3% (AC001), 63.7% (SF53) and 63.3% (I-31).

DISCUSSION

The present study showed that conidia of predatory fungi *D. flagrans*, *M. sinense* and *A. robusta* were capable of preying ($p>0.01$) Oesophagostomum spp. L_3 at the end of the experimental assay A. Isolate AC001 showed efficiency in the capture and destruction of L_3, with percent reduction of 94.4% after 7 days. These results are consistent with reports by Braga et al. 2010a that isolate AC001 was more efficient in preying *Ancylostoma ceylanicum* L_3, a geohelminth that affects dogs. Braga et al. 2010b also showed that isolate AC001 stored in silica-gel for seven years was efficient in the capture and destruction of *Haemonchus contortus* L_3. These findings confirm the efficiency and applicability of *D. flagrans* (AC001).

M. sinense (SF53) and *A. robusta* (I-31) were also shown efficient in the capture and destruction of Oesophagostomum spp L_3 with percent reductions of 92.9% and 88.3%, 7 days post-plating. Araujo et al. 2010 reported recently that *M. thaumasium* (NF34) and *A. robusta* (I-31) effectively captured and destroyed *Strongyloides westeri* L_3. These results show that the nematophagous fungi can be used to control a wide range of larval-stage nematodes.

According to Nansen et al. 1998 greater mobility of nematodes increases the stimulus for trap-formation and predation by nematophagous fungi. This was also found in our study, as we observed predation of L_3 from the first day, after 24 hours of interaction.

In assay B, conidia of isolates AC001, SF53 and I-31 were effective in reducing ($p>0.01$) Oesophagostomum L_3 of coprocultures. However, at 8 days post-plating, isolate AC001 showed the highest percent reduction (75.3%). Silva et al. 2010 reported that at the concentration of 1000 conidia, AC001 was effective in reducing *H. contortus* L_3 after eight days of coproculture, providing for an 87.5% percent reduction. Braga et al. 2009 showed that AC001 was effective in reducing cyathostomin L_3 recovered from coprocultures of horses with positive EPG. In the present study, conidia of isolates *M. sinense* (63.7%) and *A. robusta* (63.3%) in the same concentrations were also effective in reducing the Oesophagostomum L_3 in the same interval. Araújo et al. 2006 found no difference (p>0.05) between the associations and the various fungal isolates, including *M. sinense* and *A. robusta*, used to prey Cooperia sp. and Oesophagostomum sp., which agrees with our findings, indicating that nematophagous fungi can be successfully used to reduce recurrent infections.

The difference between percent reduction of larvae of the tests A and B, (AC001), 92.9% (SF53), 88.3% (I-31) and 75.3% (AC001), 63.7% (SF53), 63.3% (I-31), respectively, was probably related to the fact that the environment of the coprocultures had varying amounts of organic matter and as fungi are saprophytes part of their action was not directed towards the larvae but for organic matter.

Choosing a nematophagous fungus that produces effective results in nematode control should be considered after both in vitro and field tests. Our results suggest the need for future field studies using AC001, SF53 and I-31 against gastrointestinal nematodes in pigs.

Here, we report that conidia of *D. flagrans* (AC001), *M. sinense* (SF53) and *A. robusta* (I-31) were efficient in the capture and destruction of L_3 of Oesophagostomum spp. The fungi caused reduction in the number of L_3 recovered from the coprocultures. The main route of infection of Oesophagostomum spp. is the ingestion of infective L_3 present in the environment. Thus, the findings of this study show that these fungi can help in the control of this nematode.

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TABLES

Table 1- Daily means and standard deviation (±) of infective larvae (L3) not predated of Oesophagostomum spp by field 4 mm diameter in 2% water-agar during the period of seven days, for treatments with the fungal isolates *Duddingtonia flagrans* (AC001), *Monacrosporium sinense* (SF53), *Arthrobotrys robusta* (I-31) and in control without fungus.

Time (days)	Treatments (Means of non-predated *Oesophagostomum* L_3 per 4mm)			
	AC001	SF53	I31	Control
1	$3.86^{a\,b} \pm 2.4$	$4.98^{a} \pm 2.8$	$1.78^{b} \pm 1.0$	$11.8^{c} \pm 7.6$
2	$1.21^{a} \pm 1.26$	$1.41^{a} \pm 1.02$	$1.43^{a} \pm 0.72$	$4.15^{b} \pm 5.15$
3	$1.36^{a} \pm 1.17$	$1.76^{a} \pm 1.35$	$1.5^{a} \pm 1.2$	$2.53^{b} \pm 1.56$
4	$1.4^{a} \pm 1.2$	$1.8^{a} \pm 1.3$	$1.4^{a} \pm 1.3$	$2.5^{b} \pm 1.5$
5	$1.5^{a} \pm 1.08$	$1.68^{a} \pm 0.9$	$1.18^{a} \pm 0.8$	$5.01^{b} \pm 4.6$
6	$0.73^{a} \pm 0.8$	$0.71^{a} \pm 0.9$	$0.91^{a} \pm 0.9$	$4.81^{b} \pm 3.6$
7	$1.08^{a} \pm 1.0$	$1.8^{a} \pm 1.4$	$2.96^{a} \pm 2.7$	$11.98^{b} \pm 7.7$

Means followed by at least one common letter, in the lines, are not significantly different by the Tukey's test at a 1% probability level.

FIGURES

Fig. 1 -Means and standard deviation (bars) of infective non-predated Oesophagostomum spp. larvae recovered from 2% water-agar plates by the Baermann method on the seventh day of treatment with the following fungal isolates: *Duddingtonia flagrans* (AC001), *Monacrosporium sinense* (SF53), *Arthrobotrys robusta* (I-31) and a control group (without fungi). Asterisk denotes significant difference ($p<0.01$) between the fungus-treated group and the control - Tukey's test at a 1% probability level.

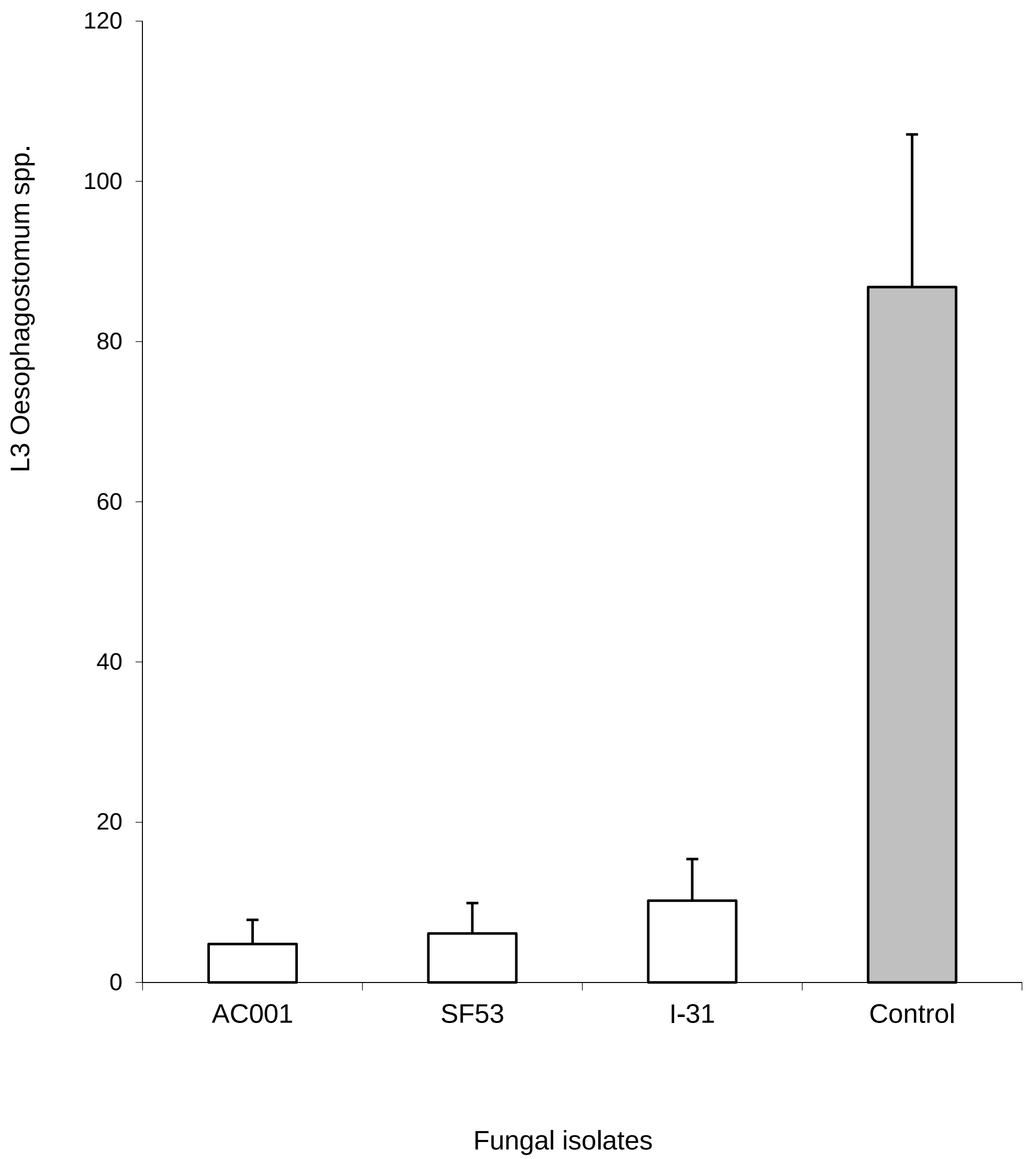

Fig. 2 - Means and standard deviation (bars) of infective non-predated Oesophagostomum spp. larvae recovered from coproculture by the Baermann method on the seventh day of treatment with the following fungal isolates: *Duddingtonia flagrans* (AC001), *Monacrosporium sinense* (SF53), *Arthrobotrys robusta* (I-31) and a control group (without fungi). Asterisk denotes significant difference (p<0.01) between the fungus-treated group and the control - Tukey's test at a 1% probability level.