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The biological control of *Ancylostoma* spp. dog infective larvae by *Duddingtonia flagrans* in a soil microcosm

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ABSTRACT

Experiments to evaluate the potential ability of the nematode-trapping fungus *Duddingtonia flagrans* (Isolate CG768) to prey on the *Ancylostoma* spp. dog infective larvae (L_3) in pasteurized soil were performed through several laboratory assays. A microcosm approach was used with increasing fungal concentrations in an inoculum of a chlamydospore water suspension. The highest fungal concentrations provide a more consistent larval reduction than the lowest concentrations, but no difference was observed from 10,000 to 25,000 chlamydospores per grain of soil. When using *D. flagrans* in a water suspension, in white rice and in milled maize, there were reductions in the larval population of 72.0%, 78.4% and 79.4%, respectively, but there was no difference between white rice and milled maize ($p < 0.05$). To evaluate the nematode control by *D. flagrans* inoculated in milled maize at 10,000 chlamydospores per grain of soil under greenhouse conditions, observations were performed at 10, 15, 20, 25 and 30 days after inoculation and the percent reduction in the larval population was 61.4%, 73.2%, 70.8%, 64.5% and 57%, respectively ($p < 0.05$). There was an inverse relationship between the number of L_3 recovered from the soil and the total days of exposure to the fungus ($p < 0.05$). These results showed that *D. flagrans* could present some potential to be used as a non-chemotherapeutic alternative for regulation of *Ancylostoma* spp. populations in the environment.

1. Introduction

The role of companion animals as reservoirs for zoonotic diseases has been recognized as a significant public health problem worldwide (Robertson et al., 2000). The gastrointestinal parasitic nematodes of the family Ancylostomatidae are blood-feeding intestinal parasites of mammalian hosts of considerable veterinary and medical importance (Schantz, 1994). Some species of the genus *Ancylostoma* only infect either humans or animals, while others, such as *A. caninum* and *A. braziliense*, are zoonotic parasites (Kalkofen, 1987; Macpherson et al., 2000). These adult hookworms live in the intestinal mucosa of infected dogs and cats, usually in tropical and subtropical regions where the climate is conducive to the development from eggs in the feces of these animals to the free-living forms in soil (Soulsby, 1982). The contaminated soil puts other pets and humans at risk of infection by hookworms and is the major source of infection in humans, especially children (Macpherson, 2005).

Given the importance of the bond between humans and animals and their relationships in modern society, environmental canine hookworm control is necessary (Schad, 1994). The regular treatment of pups and bitches with an effective anthelmintic can minimize environmental contamination by killing adult hookworms (Bowman et al.,

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2003), but rapid reinfection and the high treatment cost hamper this control effort. Furthermore, the resistance of *A. caninum* to anthelmintic therapy with pyrantel is an emerging problem that has been reported (Jackson et al., 1987; Kopp et al., 2007, 2008). Considering the fact that parasitic nematodes infecting animals spend part of their life cycle in soil (Soulsby, 1982) and that nematophagous fungi are commonly found in the soil of several ecosystems rich in organic matter worldwide (Gray, 1987), these fungi can become part of an alternative or complementary approach to effective nematode control with a reduction in anthelmintic usage (Larsen, 2000).

Nematophagous fungi are able to trap and kill nematodes and have been investigated as a means of achieving a significant reduction in the larval population in the environment, which will then result in a significant reduction in the worm burden in animals (Wolstrup et al., 1996). The species *Duddingtonia flagrans* has been reported to be the most promising biocontroller of nematodes of several domestic animals: cattle (Gronvold et al., 1993), horses (Fernández et al., 1997), pigs (Nansen et al., 1996), small ruminants (Chandrawathani et al., 2003). Its efficacy in limiting the nematode population is due to its ability to produce abundant thick-walled chlamydospores that can survive under harsh environmental conditions (anaerobic, enzymatic and thermal), such as the animal gut (Larsen, 1999). These structures can use their considerable nutritional reserve to create hyphae and adhesive three-dimensional net-type traps (Skipp et al., 2002).

Although a promising approach is to feed animals with *D. flagrans* chlamydospores (Nordbring-Hertz et al., 2006), an integrated public veterinary and medical health system should focus on the direct incorporation of this nematophagous fungus into the soil in backyards, public parks and playgrounds, especially in light of the fact that the main canine hookworm reservoir is stray dogs (Kopp et al., 2007). The incorporation of the nematophagous fungi into the soil requires the addition of organic substrate because the activity of these fungi might be stimulated by organic matter (Siddiqui and Mahmood, 1996). The biological control of canine nematodes has been neglected and there is little information on the efficacy of nematophagous fungi on the pre-parasitic forms of these hookworms in the soil. Studies reveal that *D. flagrans* is an antagonist of dog *Ancylostoma* and causes a significant reduction in the infectious larval population (Maciel et al., 2006, 2009; Carvalho et al., 2009). The present work describes the experimental results carried out to provide information on the impact of the *D. flagrans* fungus on the *Ancylostoma* spp. L_3 population in pasteurized soil under laboratory conditions, and in natural soil under greenhouse conditions, using a microcosm approach.

2. Material and methods

2.1. Fungal inoculum

Stock cultures of the previously isolated nematode-trapping fungus *D. flagrans* (Isolate CG768) were stored at 4 °C in the dark on integral rice grains in 5 mL BD Vacutainer® glass tubes (Becton Dickinson, Brazil) containing blue silica gel, similar to the conservation technique described by Smith and Onions (1983). These tubes were closed with a conventional stopper and sealed with polyvinyl chloride (PVC) transparent film in the Nematode Biocontrol Laboratory at the Federal University of Viçosa, Brazil. The identification of the fungus was confirmed on the basis of microscopic morphological features in micro-culture and trapping structures in cultures containing nematodes, as described by Cooke (1969). *D. flagrans* was selected for the present study due to its successful destruction of the *Ancylostoma* spp. L_3 observed in previous trials and its abundant production of chlamydospores.

One integral rice grain colonized by fungus was transferred from the tube to the center of a 60 mm × 15 mm Petri dish with 2% water–agar (2% WA) culture media. After seven days of incubation at 25 °C in the dark, mycelial discs 5 mm in diameter were transferred from the edges of the fungal colony to a 90 mm × 15 mm Petri dish with V8-agar media (200 mL of Campbell's V8 juice, 3 g of $CaCO_3$, 18 g of agar and 800 mL of distilled water). The new inoculated Petri dishes were kept for 15 days under the same conditions to produce *D. flagrans* chlamydospores.

Mycelial fragments and chlamydospores were harvested by gently scraping the surface of the V8-agar with a brush after the addition of 10 mL distilled water. The residual chlamydospores were then washed with 5 mL of distilled water spread over the surface of the culture medium. The fungal suspension was filtered through four layers of gauze and collected in a 50 mL Griffin glass tube to reduce mycelial fragments. Dispersant polysorbate (Tween® 80) 0.2% (v/v) was added to the suspension under agitation in a magnetic stirrer device for two minutes to disperse the chlamydospores. After careful homogenization, two aliquots of 10 μL were collected using a micropipette and placed on a Neubauer counting chamber to estimate the number of chlamydospores per milliliter of suspension.

Mass production of *D. flagrans* chlamydospores was performed with cereal grains inside 2.5 L propylene bags with biological filter. Two substrates were used: white rice and milled maize. Bags containing 300 g of a single substrate and 40 mL of distilled water were closed and sterilized for 30 min at 121 °C. The sterilized substrate in each bag was inoculated with six mycelial discs of approximately 5 mm in diameter removed from the *D. flagrans* culture edges after fungal growth in 2% WA, as previously described. The bags were closed again to allow for the growth of *D. flagrans* on the substrates for 15 days in a dark room at 25 °C. The substrate was agitated every five days until the end of the incubation period to ensure homogeneous mycelial growth.

Six samples of 1 g of colonized substrate were transferred from each bag after the incubation period to an Erlenmeyer flask containing 10 mL of distilled water plus 0.2% (v/v) dispersant polysorbate (Tween® 80) and were agitated for two minutes. The number of chlamydospores per gram of substrate was then estimated as previously described.

2.2. *Ancylostoma* spp. L_3

Ancylostoma spp. L_3 were obtained from a coproculture of feces from stray dogs containing hookworm eggs mixed with moist vermiculite for ten days at 26 °C. The feces containing hookworm eggs were identified according to Willis (1921). The active larvae were separated from the fecal cultures using the Baermann's funnel technique (Baermann, 1917) and were concentrated by gravity inside a 5 mL BD Vacutainer® glass tube (Becton Dickinson, Brazil) connected to a funnel. The sediment larvae were washed by centrifugation and resuspended in distilled water five times, at 1000 rpm, for five minutes. The supernatant was gently aspirated with a plastic pipette and discarded at the end of each centrifugation.

The method described by Barçante et al. (2003) was used to eliminate debris and obtain a clean suspension containing only viable *Ancylostoma* spp. L_3. Six aliquots containing 10 μL of the homogenized suspension were transferred to glass slides marked with longitudinal lines and covered with a glass coverslip after the addition of 10 μL of lugol solution to kill the larvae. These were then counted under a light microscope (40×). The total number of larvae in the suspension was calculated based on the average number in the aliquots. The motility of the larvae was checked under light microscopy before they were used in the assay.

2.3. Experimental procedure

In all assays, a Brazilian loamy sand soil (coarse sand = 30%; fine sand = 17%; silt = 14%; clay = 39%; organic matter = 1.6 dag/kg; phosphorus = 12.6 mg/dm^3; potassium = 37 mg/dm^3; calcium = 4.1 cmolc/dm^3; magnesium = 0.7 cmolc/dm^3; aluminum = 0 cmolc/dm^3; pH 6.4) was used. The soil was collected at a depth of 0–20 cm, sifted through a 5 mm mesh and placed in polyethylene bags. This soil was steam-pasteurized twice in a horizontal cylindrical autoclave for one hour and was then used 48 h later in *in vitro* assays. For assays under semi-natural conditions, the same soil was air-dried at room temperature for one week in order to eliminate possible *Ancylostoma* spp. larvae, as they are susceptible to dehydration (Soulsby, 1982).

2.3.1. Soil microcosm assay with different fungal concentrations

The effect of the dose of *D. flagrans* on *Ancylostoma* spp. L_3 was investigated in two *in vitro* assays. The assays were performed with 40 g of soil (initial moisture = 25%) inside 145 cm^3 transparent polypropylene pots. The soil of each pot was artificially infested with the nematode by means of a standardized suspension of 100 μL of distilled water containing approximately 1000 specimens of *Ancylostoma* spp. L_3. In a first assay, the ratios of chlamydospores inoculated per gram of soil were 500:1, 1000:1, 1500:1, 2000:1 and 2500:1; in a second assay the ratios were 5000:1, 10,000:1, 15,000:1, 20,000:1 and 25,000:1. In order to compensate for the different volumes of the chlamydospore suspension required in each pot, distilled water was added to the soil until a standardized final volume of 500 μL. Because of this, each fungus-free control pot received 500 μL of distilled water as a complementary volume. Microorganisms were mixed with soil using a disposable wooden toothpick. The pots were closed with their own plastic lids and were incubated for 15 days in a dark room at 25 °C. Every three days the pots were agitated to promote interaction between the microorganisms.

At the end of the incubation period, the larvae were recovered from the soil microcosm using the Baermann's funnel technique, as described above. A volume of 2 mL of sedimented *Ancylostoma* spp. L_3 was kept and two drops of lugol solution were added to kill them. This volume was transferred to a Peters counting slide (Peters, 1952) and the larvae were counted under a light microscope (40×).

2.3.2. Soil microcosm assay with fungal substrate

The effect of *D. flagrans* on *Ancylostoma* spp. L_3 was evaluated after the fungus was incorporated into a pasteurized soil microcosm with the culture substrate of either white rice grains or milled maize grains. The soil was artificially infested with nematodes by means of a standardized suspension of 100 μL of distilled water containing approximately 1000 specimens of *Ancylostoma* spp. L_3. A concentration of 10,000 chlamydospores per gram of soil was used due to the results of previous trials. Three test treatments and three fungus-free control treatments were performed. In one of the treatments, a suspension of *D. flagrans* chlamydospores was used, while in the other two treatments substrates which had been pre-colonized by the fungus were used. Two fungus-free control treatments contained the same amount of the sterilized substrates used in the treatments, without the nematophagous fungus.

To achieve a concentration of 10,000 chlamydospores per gram of soil, 0.69 g of milled maize or 0.88 g of white rice colonized by *D. flagrans* were added to 40 g of soil, based on the production of 577,000 and 452,000 chlamydospores per grain of these substrates, respectively. A chlamydospore suspension from the fungus grown in the V8-agar culture was added to the soil in the treatment without substrate. Equal volumes of distilled water were added to fungus-free control treatments and treatments with colonized substrate. The fungal inoculum, substrates and nematodes were mixed to the soil with a disposable wooden toothpick. The pots were closed with their own plastic lids and incubated under the same conditions as the previous trials. At the end of the period, the larvae were recovered from the soil microcosm and counted as previously described.

2.3.3. Biological control under semi-natural conditions

A technique similar to that used in a previous microcosm assay was carried out to evaluate the predatory ability of *D. flagrans* on *Ancylostoma* spp. L_3 under semi-natural conditions. The assay was performed inside 350 cm^3 transparent polypropylene pots containing 200 g of untreated soil. This soil was artificially infested with nematodes by means of a standardized suspension of 250 μL of distilled water containing approximately 5000 specimens of *Ancylostoma* spp. L_3. The fungal suspension was inoculated with 3.54 g of colonized milled maize to result in a concentration of 10,000 chlamydospores per gram of soil, due to the production of 565,000 chlamydospores per grain of milled

maize. Microorganisms were mixed with soil as described above. Two fungus-free control treatments were also performed. One of these controls contained the same amount of the sterilized substrate used in the fungal treatment, without the nematophagous fungus, and the other control had neither substrate nor fungus. The open pots were placed inside 500 cm^3 round black plastic plant pots and kept in a greenhouse, protected from direct sunlight, for a period of 30 days. The soil was irrigated every two days to prevent *Ancylostoma* larvae dehydration (Soulsby, 1982) and to allow for the germination of *D. flagrans* chlamydospores (Knox et al., 2002).

The assay was assessed beginning on the tenth day and at intervals of five days over a 30-day period. In order to recover the larvae, the soil of each pot was spread over a white paper towel sheet (22 cm × 20 cm) inside a flat plastic sieve (20 cm in diameter) and remained for 12 h on a plastic bottom plate in contact with water, initially at 41 °C. This modified Baermann's technique was used in order to improve the recovery of *Ancylostoma* spp. L_3 by improving the contact between soil and water. The larvae were collected from the plate using a wash bottle and were concentrated for six hours by gravity inside 5 mL BD Vacutainer® glass tubes (Becton Dickinson, Brazil) connected to this funnel. The larvae were then counted on a Peters counting slide, as described above.

2.4. Statistical method

The assay was carried out once, in a completely randomized design, with six replicates. Descriptive analyses of the panels were used at 95% confidence intervals when statistical inference was not possible. The data were transformed into logarithm (log_{10}) in order to equalize variances and to normalize the residuals prior to the analysis by ANOVA. The post hoc Tukey's multiple range test was performed at a 5% level of significance. Statistical inferences of the transformed data were made and back-transformed averages were presented. A regression analysis was applied to show the relationship between the variables. All statistical procedures were performed using the Statistica software system, version 7.0 (Statsoft, 2004).

The trapping ability of fungus (expressed as a percentage) was calculated as follows:

$$RP = 100 - \frac{x \cdot 100}{y}$$

In this equation, RP is the reduction percentage, x is the average number of larvae recovered from the treatment and y is the average number of larvae recovered from the fungus-free control.

3. Results

Figs. 1 and 2 present the average numbers of *Ancylostoma* spp. L_3 recovered from the soil containing five inoculum concentrations of *D. flagrans* chlamydospores. Each of the fungal concentrations reduced the larval population in relation to the fungus-free control (95% confidence interval). The highest chlamydospore concentration per gram of soil provided more consistent larval reduction

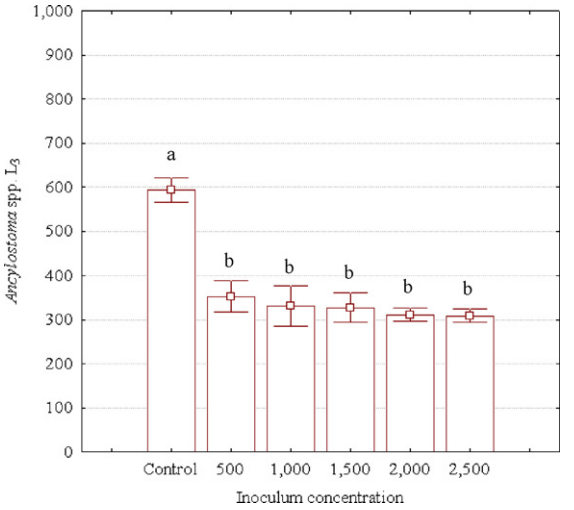

Fig. 1. Descriptive analysis of average *Ancylostoma* spp. dog infective larvae (L_3) recovered from test treatment with low inoculum concentrations of *Duddingtonia flagrans* chlamydospores in distilled water and from fungus-free control treatment after 15 days of interaction at 25 °C in a microcosm containing pasteurized soil artificially infested with 1000 L_3.

than lower concentrations, but there were no statistical differences between the five concentration levels in the low inoculum assay (Fig. 1). In the high inoculum concentration assay, fewer larvae were recovered at 5000 chlamydospores per gram of soil, which increased in the subsequent levels. Nevertheless, there was no difference between concentrations from 10,000 chlamydospores per gram of soil and above (Fig. 2). In relation to the fungus-free control, the percent reduction in the larval population at 500, 1000, 1500, 2000 and 2500 *D. flagrans* chlamydospores per gram of soil was 40.5%, 44.1%, 44.7%, 47.5% and 47.9%, respectively; at 5000, 10,000, 15,000, 20,000 and 25,000

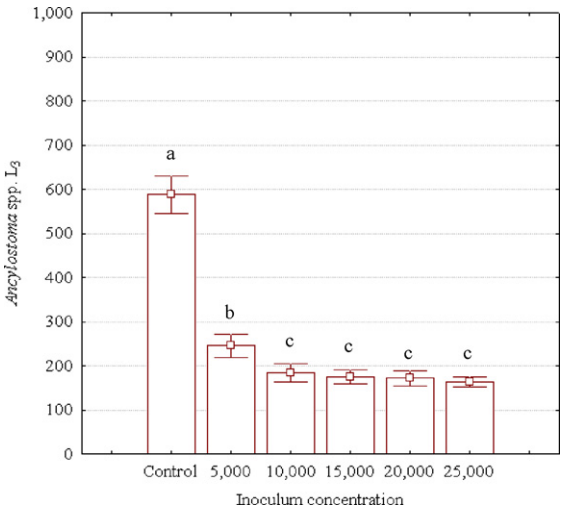

Fig. 2. Descriptive analysis at of average *Ancylostoma* spp. dog infective larvae (L_3) recovered from test treatment with high inoculum concentrations of *Duddingtonia flagrans* chlamydospores in distilled water and from fungus-free control treatment after 15 days of interaction at 25 °C in a microcosm containing pasteurized soil artificially infested with 1000 L_3.

Table 1
Average numbers, standard deviation (±) and percent reduction (%) of *Ancylostoma* spp. dog infective larvae (L_3) recovered from test treatments using *D. flagrans* in distilled water and in cereal grains (10,000 chlamydospores/g of soil) and fungus-free control treatments after 15 days of interaction at 25 °C in a microcosm containing pasteurized soil artificially infested with 1000 L_3.

Treatments	Average number	Reduction percentage (%)
D. flagrans in distilled water	$171.83^{c} \pm 5.64$	72.0
D. flagrans in white rice	$132.67^{b} \pm 13.14$	78.4
D. flagrans in milled maize	$126.33^{b} \pm 9.77$	79.4
Fungus-free white rice	$531.50^{a} \pm 57.96$	13.4
Fungus-free milled maize	$562.83^{a} \pm 63.41$	8.3
Fungus-free control	$613.67^{a} \pm 64.25$	–

Back-transformed averages followed by different superscripts in the same column are significantly different by the Tukey's test ($p < 0.05$).
Percent reduction = 100 − (treatment average × 100/control group average).

D. flagrans chlamydospores per gram of soil the percent reduction was 58.3%, 68.8%, 70.3%, 70.8% and 72.2%, respectively.

Milled maize and white rice were suitable substrates for the production of *D. flagrans* chlamydospores, but there was a statistical difference between them (with a 95% confidence interval). The fungus produced 525,347 and 640,625 chlamydospores per gram of milled maize and white rice, respectively. There was no colonization of the substrates by others microorganisms.

The number of *Ancylostoma* spp. L_3 recovered from the pasteurized soil after 15 days and the percent reduction in the larval population in comparison to the fungus-free control are presented in Table 1. There was a reduction in the number of larvae recovered from the soil in comparison to the fungus-free control ($p < 0.05$). However, there was no difference between the *D. flagrans* substrates. The percent reductions in the larval population were 72.0%, 78.4% and 79.4% for *D. flagrans* in water suspension, *D. flagrans* in white rice and *D. flagrans* in milled maize, respectively. A small reduction of 8.3% and 13.4% in the larval population was observed for fungus-free milled maize and white rice, respectively, but there was no difference between them and the fungus-free control.

The number of *Ancylostoma* spp. L_3 recovered from the natural soil over a 30-day period and the percent reduction in the larval population in comparison to the fungus-free control are presented in Table 2. The average number of larvae recovered from the treatment was larger than those recovered from the fungus-free control in all evaluations ($p < 0.05$). No statistically significant differences were found between the substrate control and fungus-free control on the 25th and 30th days ($p < 0.05$). Reductions in the nematode populations were observed when only the substrate was added to the soil, but not as much as with the colonized substrate. The percent reductions in the larval population with *D. flagrans* substrate were 61.4%, 73.2%, 70.8%, 64.5% and 57% on days 10, 15, 20, 25 and 30 of assessment, respectively, while with fungus-free substrate the values were 16.4%, 28.0%, 20.4%, 14.5% and 9.2%, respectively, on the same days of assessment. There was a linear relationship with a negative correla-

Table 2
Average numbers, standard deviation (±) and percent reduction (%) of *Ancylostoma* spp. dog infective larvae (L_3) recovered from test treatment using *D. flagrans* in milled maize (10,000 chlamydospores/g of soil) and fungus-free control treatments after 10, 15, 20, 25 and 30 days of interaction under greenhouse conditions in a microcosm containing natural soil artificially infested with 5000 L_3.

Treatments	Days				
	10	15	20	25	30
D. flagrans in milled maize	$759.00^{c} \pm 82.09$ (61.4)	$429.17^{c} \pm 69.46$ (73.2)	$319.00^{c} \pm 70.56$ (70.8)	$230.50^{b} \pm 24.73$ (64.5)	$158.83^{b} \pm 23.54$ (57)
Fungus-free milled maize	$1642.67^{b} \pm 162.91$ (16.4)	$1154.50^{b} \pm 111.55$ (28.0)	$871.17^{b} \pm 79.38$ (20.4)	$555.50^{a} \pm 51.05$ (14.5)	$335.33^{a} \pm 44.23$ (9.2)
Fungus-free control	$1964.67^{a} \pm 215.10$	$1603.33^{a} \pm 191.64$	$1094.00^{a} \pm 98.42$	$649.33^{a} \pm 53.83$	$369.33^{a} \pm 41.92$

Back-transformed averages followed by different superscripts in the same column are significantly different by the Tukey's test ($p < 0.05$).
Inside the parentheses: percent reduction = 100 − (treatment average × 100/control group average).

tion and high degree of association ($R^2 > 0.83$) between the number of recovered *Ancylostoma* spp. L_3 and the number of interaction days.Several plant-parasitic nematodes of the genera *Helicotylenchus*, *Meloidogyne*, *Mesocriconema* and *Pratylenchus* were harvested from plots without fungal inoculum, while a smaller number were harvested from those plots with *D. flagrans*. The average air temperature during the experimental period was 24.2 °C, while the minimum and maximum temperatures were 15.4 °C and 33.0 °C, respectively. The development of hyphae was observed around *D. flagrans* milled maize before the decomposition of this substrate in the soil.

4. Discussion

The results demonstrate that the nematophagous fungus *D. flagrans* reduces numbers of *Ancylostoma* spp. L_3 in the soil. The first studies with Hyphomycetes and their dispersal in the environment to reduce hookworm larvae were employed in Russian by Soprunov and Soprunova (1953) who proposed the use of spores of predaceous fungi of the genus *Didymozoophaga* (*Arthrobotrys* fide) as an anthelmintic measure to clean the soil of them. Soprunov and Tendetnik (1957) also suggested the use of predacious fungi for exterminating human hookworms larvae in mines and Soprunov (1958) observed a significant reduction of ancylostomiasis in miners after spreading considerable quantities of fungal spores in infected coal mines. Tendetnik and Davidov (1960) described a method of preparation of a powder containing spores of predacious fungi for the control of hookworm larvae in mines using moist chopped maize, glucose solution and water-melon juice. They describe the method as simple, inexpensive and effective. The addition of large amounts of fungal inoculum to soil is one of the two general applications of biological control of nematodes using nematophagous fungi (Nordbring-Hertz et al., 2006). Therefore, it is evident that the inoculum concentration of this fungus determines the predatory efficacy on *Ancylostoma* spp. L_3. The fungus may have a higher virulence against first and second stage larvae (L_1 and L_2), as Nansen et al. (1988) reported that these free-living stages are more susceptible to nematophagous fungi.

Nematode mortality is influenced by the trap density, which is directly related to the number of conidia or chlamydospores present (Gaspard and Mankau, 1987). This is in accordance with Maciel et al. (2009), who observed high larval death rates with an increase in the fungal inoculum level. The fungal predatory efficiency depends on both the ability of the nematode to induce hyphal morphogenesis in the traps and the capture efficiency of these structures (Nansen et al., 1988). The nematode larva is the most important biotic factor that leads to trap structures (Nordbring-Hertz et al., 2006). Their density is vital in stimulating the action of nematophagous fungi (Jaffee et al., 1992, 1993) such as *D. flagrans*, because when nematodes are in abundant supply, between certain threshold densities, this fungus increases trapping rates (Morgan et al., 1997). In the present study, it seems that the number of *Ancylostoma* spp. L_3 used for infesting the soil microcosm was sufficient to stimulate the food-finding mechanism of the nematophagous fungus.

The soil microcosm conditions did not mirror those existing in the environment, where several ecological interactions occur, but it did represent a biological model for the study of the predatory ability of the nematophagous fungus on target nematodes. This approach was used because it is suitable for assessing the interaction among microorganisms. With this method one can manipulate the nematode population density and the amount of the fungal inoculum, presenting sufficient replications to obtain acceptable accuracy and precision in the results (Jaffee, 1996). In addition to being experimentally convenient, the use of small volumes of soil is biologically appropriate, given the small size and limited motility of fungi and nematodes (Wiens, 1989). However, the understanding of the processes involved in the biological control of *Ancylostoma* spp. pre-parasitic forms by *D. flagrans* in an ecosystem requires a comparison between microcosm and field data. Good results were achieved in response to the inoculation of *D. flagrans* in natural soil when the microcosm was maintained under greenhouse conditions, simulating a natural ecosystem. The reduction in the phytonematode larval population indicates that *D. flagrans* could act as a biocontrol agent of these parasitic nematodes in agricultural and horticultural crops.

D. flagrans was incorporated into the soil on its growth substrate because a food base is an important factor for the establishment of fungal bio-control agents in the environment (Monfort et al., 2006), as the energy required for mycelial growth and the formation of traps can be supplied by this source of carbohydrates (Payne and Lynch, 1988; Stirling, 1991). Thus, with an energy source readily available, the fungus probably would not have nutritional limitations to colonize the soil profile and form structures to capture nematodes (Dias and Ferraz, 1994). However, fungistasis is identified as a major limiting factor for survival, and the soil microbiota has been reported as one of the main factors that affect the survival of nematophagous fungi because it can inhibit its germination, growth and activity (Kerry, 1984). The results of this study revealed that the possible presence of other microorganisms in unpasteurized soil did not affect the *D. flagrans* trapping efficacy, since there was consistent larval control. Therefore, a reasonable interpretation is that *D. flagrans* used the milled maize to establish itself in the soil. This was possible because predatory adhesive network forming fungi are good saprophytes which feed on other nutrient sources in the absence of nematodes to ensure their survival in the soil (Nordbring-Hertz, 1988). Furthermore, the *D. flagrans* chlamydospores used as the fungal inoculum are viable for an extended period in soil under unfavourable environments (Sanyal and Mukhopadhyaya, 2003) due to their ability to germinate and form mycelia and traps in response to nematode activity (Skipp et al., 2002). The reduction in the larval population in plots with uncolonized milled maize may have been caused by the stimulation of the activity of nematophagous microorganisms present in the untreated soil. Therefore, the natural occurrence of indigenous microorganisms demonstrates that the manipulation of the environment to increase existing populations

of nematophagous fungi may also be feasible (Waghorn et al., 2002).

The induction of traps in nematophagous fungi is also influenced by environmental factors, such as pH, light, moisture (Nordbring-Hertz, 1973) and temperature (Bogus et al., 2005). Especially, the soil temperature may be the main biologically significant physical variable (Stirling, 1991) because the optimum temperature for nematophagous fungi varies and each species affects the percentage of arrested larvae (Morgan et al., 1997). The air temperatures during the experimental period were vital for the activity of *D. flagrans* in the soil as evidenced by the low number of *Ancylostoma* spp. L_3 recovered from the treated plots. According to Gronvold et al. (1996) and Morgan et al. (1997), the optimal growth temperature for *D. flagrans* ranges from 25 to 33 °C. At temperatures lower than 25 °C, it grows slowly (Larsen, 1991); growth is limited at 35 °C and stops completely at 37 °C (Peloille, 1991). Despite the high temperature regime that exceeded 30 °C inside the greenhouse, the real temperature in the soil was lower than the air temperatures recorded. Gronvold et al. (1999) reported that *D. flagrans* produces a higher number of traps at 30 °C, compared to 20 °C. At 10 °C, it has a slower development without decreasing its trapping capacity, though this can be compromised at temperatures persistently below 10 °C (Fernández et al., 1999). This trapping capacity may also be dependent on the duration of exposure to high temperatures (Kahn et al., 2007). In addition, the effect of temperature fluctuation on the trapping efficacy of *D. flagrans* demonstrated a dependence on the maintenance of an appropriate temperature, with a reduction in the larva population between 15 and 25 °C (Fernández et al., 1999). Based on the reports presented above, it is reasonable to state that the temperature maintained during the experimental period, between 14.5 and 33 °C, contributed to optimal fungal activity.

Factors such as the selection of an isolate with rapid growth and abundant sporulation that is pathogenic to the target organism, as well as the use of an organic substrate that is minimally processed, inexpensive, easily obtained and practical were taken into account in the present study, based on the reports by Samsinakova et al. (1981). The propylene bags with a biological filter were a feasible and practical method to obtain a large number of *D. flagrans* chlamydospores in a short time, providing an aseptic environment for fungal growth. Furthermore, white rice and milled maize grains were suitable substrates for the production of this fungus' inoculum, which is consistent with reports from Waller et al. (2001). However, other cereal grains such as wheat, sorghum and barley are also good organic substrates for the production of *D. flagrans* chlamydospores (Sanyal, 2000; Sanyal and Mukhopadhyaya, 2003).

The continued recovery of *Ancylostoma* spp. L_3 until the last day of evaluation revealed the survivability of this nematode in the soil, which is facilitated by the larval arrest at the L_3 stage, according to Datu et al. (2008). One of the probable reasons for the persistence of larvae in the soil microcosm is the fact that the predatory phase of nematophagous fungi continues only until their nutritional requirement is met (Hayes and Blackburn, 1966). Moreover, the activity of nematophagous fungi in the environment tends to be short-lived due to the ecological factors that limit their population in the field (Cooke and Satchuthananthavale, 1968). Nonetheless, a new incorporation of *D. flagrans* inoculum could possibly completely eliminate *Ancylostoma* spp. larvae in the microcosm. However, the survival of a few parasitic nematode larvae is acceptable because the objective of the biological control is to reduce the population of the larvae in the environment to a level that prevents adverse effects in the host animals, but that is still sufficient to stimulate a naturally acquired active immunity to them (Larsen et al., 1997). The *D. flagrans* nematophagous activity is not specific to the target nematode. Therefore, the nematode community or indigenous microorganisms in the soil ecosystem may be disturbed by the introduction of large amounts of this fungus. However, in several studies with *D. flagrans*, no damage was observed to earthworms (Gronvold et al., 2000), free-living nematodes or microarthropods (Knox et al., 2002).

Currently, there are no restrictions to the use of nematophagous fungi in the control of pre-parasitic stages of dog nematodes in the environment. However, the biological control does not preclude the use of anthelmintics, since nematophagous fungi do not have any effect on nematodes after they have infected a host (Jackson and Miller, 2006). Hence, chemical treatment is considered necessary in addition to the non-chemotherapeutic approach to animal health. This study demonstrated that the nematode-trapping fungus *D. flagrans* (Isolate CG768) at 10,000 chlamydospores per grain of soil presented satisfactory results reducing the numbers of *Ancylostoma* spp. L_3 in a soil microcosm under greenhouse conditions. Although there are no guarantees that this concentration of *D. flagrans* will cause satisfactory damage to the dog *Ancylostoma* population in the field, the present results provide considerable encouragement for further research under environmental conditions.

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References

Baermann, G., 1917. Eine einfache methode zur auffindung von *Ancylostomum* (Nematoden) larven in erdproben, Gennesk. Tijds. Ned. Ind. 57, 131–137.

Barçante, J.M.P., Barçante, T.A., Dias, S.R.C., Vieira, L.Q., Lima, W.S., Negrão-Corrêa, D., 2003. A method to obtain axenic *Angiostrongylus vasorum* first-stage larvae from dog feces. Parasitol. Res. 89, 89–93.

Bogus, M.I., Czygier, M., Kedra, E., Samborski, J., 2005. *In vitro* assessment of the influence of nutrition and temperature on growing rates of five *Duddingtonia flagrans* isolates, their insecticidal properties and ability to impair *Heligmosomoides polygyrus* motility. Exp. Parasitol. 109, 115–123.

Bowman, D.D., Lynn, R.C., Eberhard, M.L., Georgi, J.R., 2003. Georgis' Parasitology for Veterinarians. Saunders, St. Louis, 422 pp.

Carvalho, R.O., Araújo, J.V., Braga, F.R., Araujo, J.M., Silva, A.R., Tavela, A.O., 2009. Predatory activity of nematophagous fungi on infective larvae of *Ancylostoma* sp.: evaluation *in vitro* and after passing through the gastrointestinal tract of dogs. J. Helminthol. 83, 231–236.

Chandrawathani, P., Jamnah, O., Waller, P.J., Larsen, M., Gillespie, A., Zahari, W.M., 2003. Biological control of nematode parasites of small ruminants in Malaysia using the nematophagous fungus *Duddingtonia flagrans*. Vet. Parasitol. 117, 173–183.

Cooke, R.C., 1969. Two nematode-trapping Hyphomycetes, *Duddingtonia flagrans* gen. et comb. nov. and *Monacrosporium mutabilis* sp. nov. Trans. Br. Mycol. Soc. 53, 315–319.

Cooke, R.C., Satchuthananthavale, V., 1968. Sensitivity to mycostasis of nematode-trapping hyphomycetes. Trans. Br. Mycol. Soc. 51, 555–561.

Datu, B.J.D., Gasser, R.B., Nagaraj, S.H., Ong, E.K., O'Donoghue, P., McInnes, R., Ranganathan, S., Loukas, A., 2008. Transcriptional changes in the hookworm, *Ancylostoma caninum*, during the transition from a free-living to a parasitic larva. PLoS Negl. Trop. Dis. 1, 1–15.

Dias, W.P., Ferraz, S., 1994. Avaliação de espécies de *Arthrobotrys* para o controle de *Meloidogyne incognita*. Fitopatol. Bras. 19, 189–192.

Fernández, A.S., Larsen, M., Henningsen, E., Nansen, P., Gronvold, J., Bjorn, H., Wolstrup, J., 1999. Effect of *Duddingtonia flagrans* against *Ostertagia ostertagi* in cattle grazing at different stocking rates. Parasitology 119, 105–111.

Fernández, A.S., Larsen, M., Nansen, P., Gronvold, J., Henriksen, S.A., Wolstrup, J., 1997. Effect of the nematode-trapping fungus *Duddingtonia flagrans* on the free-living stages of horse parasitic nematodes; a plot study. Vet. Parasitol. 73, 257–266.

Gaspard, J.T., Mankau, R., 1987. Density-dependence and host-specificity of the nematode-trapping fungus *Monacrosporium ellipsosporum*. Rev. Nematol. 10, 241–246.

Gray, N.F., 1987. Nematophagous fungi with particular reference to their ecology. Biol. Rev. 62, 245–304.

Gronvold, J., Wolstrup, J., Larsen, M., Henriksen, S.A., Nansen, P., 1993. Biological control of *Ostertagia ostertagi* by feeding selected nematode-trapping fungi to calves. J. Helminthol. 67, 31–36.

Gronvold, J., Nansen, P., Henriksen, S.A., Larsen, M., Wolstrup, J., Bresciani, J., Rawat, H., Fribert, L., 1996. Induction of traps by *Ostertagia ostertagi* larvae, chlamydospore production and growth rate in the nematode-trapping fungus *Duddingtonia flagrans*. J. Helmintol. 70, 291–297.

Gronvold, J., Wolstrup, J., Nansen, P., Larsen, M., Bjorn, H., 2000. Absence of obvious short term impact of the nematode-trapping fungus *Duddingtonia flagrans* on survival and growth of the earthworm *Aporrectodea longa*. Acta Vet. Scand. 41, 147–151.

Gronvold, J., Wolstrup, J., Nansen, P., Larsen, M., Henriksen, S.A., Bjorn, H., Kirchheiner, K., Lassen, K., Rawat, H., Kristiansen, H.L., 1999. Biotic and abiotic factors influencing growth rate and production of traps by the nematode trapping fungus *Duddingtonia flagrans* when induced by *Cooperia oncophora* larvae. J. Helminthol. 73, 129–136.

Hayes, W.A., Blackburn, F., 1966. Studies on the nutrition of *Arthrobotrys oligospora* Fres. and *Arthrbotrys robusta* Dudd. Ann. Appl. Biol. 58, 51–60.

Jackson, F., Miller, J., 2006. Alternative approaches to control—quo vadit? Vet. Parasitol. 139, 371–384.

Jackson, R., Lance, D., Townsend, K., 1987. Isolation of anthelmintic resistant *Ancylostoma caninum*. N. Z. Vet. J. 35, 215–216.

Jaffee, B., Phillips, R., Muldoon, A., Mangel, M., 1992. Density-dependent host-pathogen dynamics in soil microcosms. Ecology 73, 495–506.

Jaffee, B.A., 1996. Soil microcosms and the population biology of nematophagous fungi. Ecology 77, 690–693.

Jaffee, B.A., Tedford, E.C., Muldoon, A.E., 1993. Tests of density-dependent parasitism of nematodes by nematode-trapping and endoparasitic fungi. Biol. Control 3, 329–336.

Kalkofen, U.P., 1987. Hookworm in dogs and cats. Vet. Clin. North Am. Small Anim. Pract. 17, 1341–1354.

Kahn, L.P., Norman, T.M., Walkden-Brown, S.W., Crampton, A., O'Connor, L.J., 2007. Trapping efficacy of *Duddingtonia flagrans* against *Haemonchus contortus* at temperatures existing at lambing in Australia. Vet. Parasitol. 146, 83–89.

Kerry, B.R., 1984. Nematophagous fungi and regulation of nematode populations in soil. Helmithol. Abst. 53, 1–14.

Knox, M.R., Josh, P.F., Anderson, L.J., 2002. Deployment of *Duddingtonia flagrans* in an improved pasture system: dispersal, persistence, and effects on free-living soil nematodes and microarthropods. Biol. Control 24, 176–182.

Kopp, S.R., Coleman, G.T., McCarthy, J.S., Kotze, A.C., 2008. Application of *in vitro* anthelmintic sensitivity assays to canine parasitology: detecting resistance to pyrantel in *Ancylostoma caninum*. Vet. Parasitol. 152, 284–293.

Kopp, S.R., Kotze, A.C., McCarthy, J.S., Coleman, G.T., 2007. High-level pyrantel resistance in the hookworm *Ancylostoma caninum*. Vet. Parasitol. 143, 299–304.

Larsen, M., 1999. Biological control of helminths. Int. J. Parasitol. 29, 139–146.

Larsen, M., 2000. Prospects for controlling animal parasitic nematodes by predacious micro fungi. Parasitology 120, 121–131.

Larsen, M., Nansen, P., Gronvold, J., Wolstrup, J., Henriksen, S.A., 1997. Biological control of gastro-intestinal nematodes—facts, future or fiction? Vet. Parasitol. 72, 479–492.

Larsen, M., 1991. Studies on the capacity of microfungi to destroy animal parasitic nematode. Thesis (Ph.D.). The Royal Veterinary and Agricultural University, Copenhagen, Denmark, 61 pp.

Maciel, A.S., Araújo, J.V., Campos, A.K., Lopes, E.A., Freitas, L.G., 2009. Predation of *Ancylostoma* spp. dog infective larvae by nematophagous fungi in different conidial concentrations. Vet. Parasitol. 161, 239–247.

Maciel, A.S., Araújo, J.V., Cecon, P.R., 2006. Atividade predatoria *in vitro* dos fungos *Arthrobotrys robusta*, *Duddingtonia flagrans* e *Monacrosporium thaumasium* sobre larvas infectantes de *Ancylostoma* spp. de cães. Rev. Bras. Parasitol. Vet. 15, 71–75.

Macpherson, C.N.L., 2005. Human behaviour and the epidemiology of parasitic zoonoses. Int. J. Parasitol. 35, 1319–1331.

Macpherson, C.N.L., Meslin, F.X., Wandeler, A., 2000. Dogs, Zoonoses and Public Health. CAB International Publishing, Wallingford, 400 pp.

Monfort, E., Lopez-Llorca, L.V., Jansson, H.B., Salinas, J., 2006. *In vitro* soil receptivity assays to egg-parasitic nematophagous fungi. Mycol. Prog. 5, 18–23.

Morgan, M., Behnke, J.M., Lucas, J.A., Peberdy, J.F., 1997. *In vitro* assessment of the influence of nutrition, temperature and larval density on trapping of the infective larvae of *Heligmosomoides polygyrys* by *Arthrobotrys oligospora, Duddingtonia flagrans* and *Monacrosporium megalosporum*. Parasitology 115, 303–310.

Nansen, P., Gronvold, J., Henriksen, S.A., Wolstrup, J., 1988. Interactions between the predacious fungus *Arthrobotrys oligospora* and third-stage larvae of a series of animal-parasitic nematodes. Vet. Parasitol. 26, 329–337.

Nansen, P., Larsen, M., Roepstorff, A., Gronvold, J., Wolstrup, J., Henriksen, S.A., 1996. Control of *Oesophagostomum dentatum* and *Hyostrongylus rubidus* in outdoor-reared pigs through daily feeding with the microfungus *Duddingtonia flagrans*. Parasitol. Res. 82, 580–584.

Nordbring-Hertz, B., 1973. Peptide-induced morphogenesis in the nematode trapping fungus *Arthrobotrys oligospora*. Physiol. Plant 29, 223–233.

Nordbring-Hertz, B., 1988. Nemathophagus fungi: strategies for nematode exploitation and for survival. Microbiol. Sci. 5, 108–168.

Nordbring-Hertz, B., Jansson, H.B., Tunlid, A., 2006. Nematophagous fungi. Encyclopedia of Life Sciences. John Wiley & Sons, Chichester, pp. 1–11.

Payne, C.C., Lynch, J.M., 1988. Biological control. In: Lynch, J.M., Hobbie, J.E. (Eds.), Concepts and Applications in Microbial Ecology. Blackwell Scientific Publications, Oxford, pp. 261–287.

Peloille, M., 1991. Selection of nematode-trapping fungi to use in biological control. In: Kerry, B.R., Crump, D.H. (Eds.), Integrated Control of Soil Pests. Methods for Studying Nematophagous Fungi. IOBC/WPRS Bulletin, XIV/2, pp. 13–17.

Peters, B.G., 1952. Toxicity tests with vinegar eelworm. Counting and culturing. J. Helminthol. 26, 97–110.

Robertson, I.D., Irwin, P.J., Lymbery, A.J., Thompson, R.C.A., 2000. The role of companion animals in the emergence of parasitic zoonoses. Int. J. Parasitol. 30, 1369–1377.

Samsinakova, A., Kalalova, S., Vlcek, V., Kybal, J., 1981. Mass production of *Beauveria bassiana* for regulation of *Leptinotarsa decemlineata* populations. J. Invert. Pathol. 38, 169–174.

Sanyal, P.K., 2000. Screening for Indian isolates of predacious fungi for use in biological control against nematode parasites of ruminants. Vet. Res. Commun. 24, 55–62.

Sanyal, P.K., Mukhopadhyaya, P.N., 2003. Top dressing of feeding with desiccated chlamydospores of *Duddingtonia flagrans* for biological control of the pre-parasitic stages of ovine *Haemonchus contortus*. Vet. Res. Commun. 27, 381–390.

Schad, G.A., 1994. Hookworms: pets to humans. Ann. Int. Med. 120, 434–435.

Schantz, P.M., 1994. Of worms, dogs, and human hosts: continuing challenges for veterinarians in prevention of human disease. J. Am. Vet. Med. Assoc. 204, 1023–1028.

Siddiqui, Z.A., Mahmood, I., 1996. Biological control of plant parasitic nematodes by fungi: a review. Biores. Technol. 58, 229–239.

Skipp, R.A., Yeates, G.W., Chen, L.Y., Glare, T.R., 2002. Occurrence, morphological characteristics and ribotyping of New Zealand isolates of

Duddingtonia flagrans, a candidate for biocontrol of animal parasitic nematodes. N. Z. J. Agric. Res. 45, 187–196.
Smith, D., Onions, A.H.S., 1983. The Preservation and Maintenance of Living Fungi. Commonwealth Mycological Institute, Surrey, 51 pp.
Soprunov, F.F., 1958. Predacious Hyphomycetes and their Application in the Control of Pathogenic Nematodes, Academy of Sciences of Turkmen, Ashkhabad. (Translation from Russian.) Israel Program for Scientific Translations, Washington, 292 pp.
Soprunov, F.F., Soprunova, N.Y., 1953. Cleansing the Soil of Pathogenic Nematodes and their Larvae by Means of the Spore of Carnivorous Fungi of the Genus *Didymozoophaga* (in Russian), Academy of Sciences of Turkmen, Ashkhabad. (Translation from Russian.) Israel Program for Scientific Translations, Washington, 167 pp.
Soulsby, E.J.L., 1982. Helminths, Arthropods and Protozoa of Domesticated Animals. Baillière Tindall, London, 806 pp.
Statsoft, 2004. Statistica for Windows. Computer Program Manual. Version 7.0. Statsoft, Tulsa, http://www.statsoft.com.
Stirling, G.R., 1991. Biological Control of Plant Parasitic Nematodes: Progress, Problems and Prospects. CAB International, Wallingford, 282 pp.
Soprunov, F.F., Tendetnik, Y.Y., 1957. Results of an experiment in biological control of ancylostomiasis in coal mines. Med. Parasitol. 26, 602–612.
Tendetnik, Y.Y., Davidov, L.N., 1960. Preparation of a Powder Containing Spores of Predacious Fungi, vol. 5. Trudy Instituta Zoologii i Parazitologii. Akademiya Nauk Turkmenskoi SSR, pp. 189–193.
Waghorn, T.S., Leathwick, D.M., Chen, L.Y., Gray, R.A.J., Skipp, R.A., 2002. Influence of nematophagous fungi, earthworms and dung burial on development of the free-living stages of *Ostertagia* (*Teladorsagia*) *circumcincta* in New Zealand. Vet. Parasitol. 104, 119–129.
Waller, P.J., Knox, M.R., Faedo, M., 2001. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: feeding and block studies with *Duddingtonia flagrans*. Vet. Parasitol. 102, 321–330.
Wiens, J.A., 1989. Spatial scaling in ecology. Funct. Ecol. 3, 385–397.
Willis, I.I., 1921. A simple levitation method for the detection of hookworm ova. Med. J. Aust. 8, 375–376.
Wolstrup, J., Nansen, P., Gronvold, J., Henriksen, S.A., Larsen, M., 1996. Towards practical biological control of parasitic nematodes in domestic animals. J. Nematol. 28, 129–132.