RAFAELA CAROLINA LOPES ASSIS

VIABILIDADE DOS FUNGOS PREDADORES *Monacrosporium thaumasium* E *Duddingtonia flagrans* E DA MOXIDECTINA SOBRE NEMATOIDES GASTRINTESTINAIS DE BOVINOS DE CORTE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 25 de julho de 2013.

Profª. Márcia Cristina Cury

Profª. Isabele da Costa Angelo

Prof. Fábio Ribeiro Braga
(Coorientador)

Profª. Maria Verônica de Souza

Prof. Jackson Victor de Araújo
(Orientador)

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RESUMO

ASSIS, Rafaela Carolina Lopes, D.Sc., Universidade Federal de Viçosa, julho de 2013. **Viabilidade dos fungos predadores *Monacrosporium thaumasium* e *Duddingtonia flagrans* e da moxidectina sobre nematoides gastrintestinais de bovinos de corte.** Orientador: Jackson Victor de Araújo. Coorientador: Fábio Ribeiro Braga.

Dois diferentes fungos predadores de nematóides *Duddingtonia flagrans* (AC001) e *Monacrosporium thaumasium* (isolado NF34A) e o anti-helmíntico moxidectin foram avaliados no controle de nematóides gastrointestinais de bovinos de corte a campo. Quatro grupos contendo dez bezerros da raça Nelore cada, machos, com seis meses de idade, foram mantidos na fazenda experimental da Universidade Federal de Viçosa, no *campus* Florestal, Minas Gerais, em piquetes de *Brachiaria decumbens* por 12 meses, de maio de 2010 a junho de 2011. Cada animal do grupo A recebeu 1g/10 kg de peso vivo (PV) de pellets de alginato de sódio de *D. flagrans* (0,2 g de fungo/10 kg de PV), duas vezes por semana, durante 12 meses. No grupo B, cada animal recebeu 1g/10 Kg de PV de pellets de alginato de sódio de *M. thaumasium*, duas vezes por semana, durante 12 meses. No grupo C, cada bezerro recebeu 1mL/50Kg de PV do anti-helmíntico moxidectin a 1% em maio, julho e setembro de 2010. Animais do grupo controle (grupo D) não receberam tratamento. Na contagem de ovos por grama de fezes (OPG) os grupos A e B apresentaram redução de 56,7% e 47,8%, respectivamente em relação ao grupo controle. O moxidectin reduziu o OPG em 93,8% nos primeiros seis meses (período de aplicação), mas nos últimos seis meses a redução foi de 12,4%, indicando reinfecção por nematóides. A redução de L3 na pastagem nas distâncias de 20 e 20-40 cm do bolo fecal foi de 64,5 e 73% para o fungo *D. flagrans* e 47,3 e 58% para o *M. thaumasium*. O controle químico com moxidectin não foi capaz de reduzir o número de larvas na pastagem. O isolado fúngico de *D. flagrans* (AC001) apresentou maior redução de OPG e de L3 na pastagem quando comparado ao *M. thaumasium* (NF34A). O tratamento do gado de corte com os pellets contendo fungos nematófagos e com o moxidectin pode ser usado de forma integrada como alternativa de controle de nematodioses; no entanto, o controle biológico é mais eficiente do que o controle químico, pois atua em longo prazo e permite melhora no ganho de peso dos bezerros, fator essencial à bovinocultura de corte.

ABSTRACT

ASSIS, Rafaela Carolina Lopes, D.Sc., Universidade Federal de Viçosa, July, 2013. **Viability of fungi predators *Monacrosporium thaumasium* and *Duddingtonia flagrans* and moxidectin on gastrointestinal nematodes of beef cattle.** Adviser: Jackson Victor de Araújo. Co-adviser: Fábio Ribeiro Braga.

Sodium alginate pellets of two different nematode predatory fungus *D. flagrans* and *Monacrosporium thaumasium* (biologic control) and Moxidectin (chemical control) were evaluated in the control of bulls gastrointestinal nematodiasis. Four groups of ten six month-old male Nellore calves were kept in paddocks of *Brachiaria decumbens* for 12 months. Each animal of group A received 1g/10 kg of body weight (b.w.) of pellets of *D. flagrans* (0.2 g of fungus/10 kg b.w.) twice a week, for 12 months. In group B, each animal received 1g/10 Kg of body weight of *M. thaumasium* pellets, twice a week, for 12 months. In group C each animal received 1 mL/50 Kg body weight of the anthelmintic moxidectin 1% in May, July and September 2010. Animals of the group control (group D) received no treatment. In the analysis of fecal egg count (EPG) of groups A and B were 56.7% and 47.8% smaller than control group ($p<0.05$). The Moxidectin reduced by 93.8% in the first six months of the study (period of application) and 12.4% in the last six months, indicating re-infection by nematodes. The biologic control maintains a low EPG in long-term while chemical control results in a short-term effect. The percentage reduction of L3 in relation to the group control in the distances of up to 20 and 20-40 cm from the fecal pat was 64.5% and 73%, respectively, for group A, group B showed percentage reductions of 47.3% and 58%, in the same distances. The fungal isolate of *D. flagrans* (AC001) showed greater reduction of EPG and L3 on pasture when compared to *M. thaumasium*. Treatment of bulls with pellets containing the nematophagous fungus and moxidectin can be used integrated as an alternative treatment of bovine gastrointestinal nematodiasis; however, biologic was more efficient than chemical control for a long-term and allows improvement in weight gain of calves, an essential factor for beef cattle.

1. INTRODUÇÃO GERAL

A criação de ruminantes é uma atividade de relevante importância sócio econômica para o Brasil. Os bovinos, caprinos e ovinos representam uma das principais fontes proteicas para a população, sendo que a produção de carne, lã, leite e derivados vêm crescendo a cada ano para atender a demanda do mercado consumidor (ANUALPEC, 2012).

A bovinocultura é um dos principais destaques do agronegócio brasileiro no cenário mundial. O Brasil possui o segundo maior rebanho efetivo do mundo, com cerca de 200 milhões de cabeças. Além disso, desde 2004, assumiu a liderança nas exportações, com um quinto da carne comercializada internacionalmente e vendas em mais de 180 países. Segundo o Ministério da Agricultura, até 2020, a expectativa é que a produção nacional de carne bovina suprirá 44,5% do mercado mundial. Essa estimativa indica que o Brasil manterá posição de primeiro exportador mundial de carne bovina (MAPA, 2012).

No Brasil, a maior parte da criação de bovinos é feita em regime de pasto total ou parcial, o que causa constantes infecções pelos parasitos presentes nas pastagens. Assim, as pastagens são consideradas a principal via de infecção desses parasitos para os bovinos (DIAS et al., 2007).

As condições ambientais são fatores que influenciam diretamente nas condições da pastagem e, por sua vez, oferecem as condições favoráveis para que os estádios de vida livre dos nematoides gastrintestinais alcancem o estádio infectante (AMARANTE et al., 1996).

Os prejuízos causados pelas infecções parasitárias envolvem queda da produção, retardo no crescimento do animal, gastos com recursos terapêuticos e até na morte dos animais (MOTA et al., 2003).

Faz-se necessário conhecer a epidemiologia dos parasitos e as suas interações com o hospedeiro em determinado ambiente, condição climática e sistema produtivo, sendo estes requerimentos indispensáveis para estabelecer um sistema de controle efetivo. A falta de tais informações pode levar a utilização inadequada dos tratamentos anti-helmínticos, ocasionando a resistência a essas drogas (ARAÚJO et al., 2004a).

A resistência aos anti-helmínticos, como já observado por décadas no mercado de ovinos, começa a ser um fator preocupante também para bovinos (BORDIN, 2004).

Dentre os parasitos gastrointestinais, no sudeste do Brasil, *Cooperia* e *Haemonchus*, são os nematoides mais prevalentes em bovinos, seguidos pelo gênero *Oesophagostomum*, que aparece em terceiro lugar de destaque (ARAÚJO et al., 1998). Do gênero *Haemonchus*, *Haemonchus placei* é considerado a espécie de maior ocorrência no Brasil, com altas taxas de morbidade. É parasito hematófago que produz lesões na mucosa do abomaso e pode causar perdas de sangue e plasma para a luz intestinal. Infecções maciças podem levar a hipoproteinemia e anemia aguda em animais jovens, causando a morte (NISHI et al., 2002).

Frente às perdas ocasionadas pelas parasitoses, o custo do tratamento com químicos e a crescente resistência dos parasitos aos compostos anti-helmínticos, prevê-se novas alternativas de controle dessas infecções (MOTA et al., 2003).

Nas pesquisas alternativas para o controle das helmintoses de ruminantes destacam-se o desenvolvimento de vacinas, manejo de pastagens, seleção de animais geneticamente resistentes aos parasitos e a prática do controle biológico, onde antagonistas naturais atuam na redução de uma população de pragas que causam perdas econômicas significativas (ARAÚJO & RIBEIRO, 2003). Dentre os mais variados antagonistas de nematóides, encontram-se organismos como fungos, bactérias, protozoários, vírus, entre outros, sendo os fungos nematófagos aqueles que apresentam melhor desempenho em pesquisa de controle biológico de nematoides (MACIEL et al., 2006), destacando-se dentre estes, os fungos predadores dos gêneros *Arthrobotrys*, *Duddingtonia* e *Monacrosporium* (ARAÚJO et al., 2004b).

Fungos nematófagos são organismos saprófitas mundialmente estudados, com capacidade de predar nematoides pela produção armadilhas ao longo de suas hifas (MOTA et al., 2002) exibem redução efetiva na população de nematoides em experimentos laboratoriais e podem promover eficácia em experimentos realizados a campo (ARAÚJO et al., 1998). Esses fungos pertencem a um grupo heterogêneo que utilizam nematóides como fonte principal ou adicional de nutrientes. São encontrados em todo o mundo, em diferentes habitats, sendo frequentemente encontrados em ambientes ricos em material orgânico (LARSEN, 2000) e temperaturas que podem variar de 20 a 30°C (FERNÁNDEZ et al., 1999). Eles são conhecidos como fungos destruidores de nematoides e são catalogados em mais de 150 espécies (BARRON, 1977). São divididos em três grupos denominados endoparasitas, predadores e oportunistas, mas a maioria das espécies é classificada como fungos predadores de nematóides. Estes produzem estruturas em forma de aneis constritores e não constritores, hifas, botões e redes tridimensionais adesivas ao longo do micélio. Depois de aprisionarem o nematóide, segue-se a penetração das hifas na cutícula do nematóide, onde ocorre o crescimento das hifas e digestão dos conteúdos internos (LARSEN et al, 1999; ARAÚJO et al., 2004a).

Para um fungo ser considerado como um agente promissor no controle biológico é necessário ter habilidade de passar pelo trato gastrintestinal do ruminante para ser disseminado nas fezes do animal. Fungos das espécies *Arthrobotrys robusta*, *Duddingtonia flagrans* e *Monacrosporium thaumasium* tem atividade predatória comprovada contra as larvas dos helmintos gastrintestinais de bovinos, sendo capazes de resistir à passagem gastrintestinal (GRONVOLD et al., 1993 e 2004; LARSEN et al., 1995; ARAÚJO et al., 1999; RODRIGUES et al., 2001; MOTA et al., 2003).

Os fungos nematófagos vêm se apresentando como alternativa no controle desses nematóides parasitas em pastagens, reduzindo grande parte das infecções e contribuindo no controle desses parasitos nos animais (GRONVOLD et al, 1996 e 2004).

No Brasil, os estudos pioneiros demonstrando a ação de fungos nematófagos sobre larvas infectantes de *Haemonchus placei*, parasitos de bovinos, foram desenvolvidos por Araújo et al. (1992). Outros trabalhos também comprovaram a ação dos fungos nematófagos sobre nematoides gastrintestinais de bovinos relatando ser alternativa promissora no controle biológico (GRONVOLD et al., 1993; LARSEN et al., 1999; MOTA et al., 2002)

Em alguns laboratórios de pesquisa, formulações a base de alginato de sódio tem sido avaliadas, demonstrando bons resultados em condições laboratoriais e a campo (ARAÚJO et al., 2000).

2. OBJETIVOS

2.1. Objetivo geral

- Avaliar a ação dos fungos *Monacrosporium thaumasium* e *Duddingtonia flagrans* em pellets de alginato de sódio e do anti-helmíntico moxidectin 1% (Cydectin – Fortdodge®) no controle das nematodioses gastrintestinais de bovinos de corte.

2.2. Objetivos específicos

- Realizar contagem de ovos por grama de fezes e de larvas recuperadas em pastagem dos animais do grupo controle e dos grupos tratados com *Duddingtonia flagrans*, *Monacrosporium thaumasium* e com o anti-helmíntico moxidectin a 1% (Cydectin – Fortdodge®);

- Determinar o tratamento mais promissor no controle das nematodioses gastrintestinais de bovinos;

- Acompanhar o peso dos animais ao longo do tratamento;

- Constatar a existência de interação entre os fungos *D. flagrans* e *M. thaumasium* e as larvas infectantes de *Haemonchus placei* por meio de Microscopia Eletrônica de Varredura.

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CAPÍTULO 1 – *Experimental Parasitology* 132 (2012) 373-377

Biological control of trichostrongyles in beef cattle by the nematophagous fungus *Duddingtonia flagrans* in tropical southeastern Brazil

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Abstract

The efficacy of a fungal formulation based on the nematophagous fungus *Duddingtonia flagrans* was assessed in the control of cattle trichostrongyles. Twenty male Nellore calves, six-month-old, divided in two groups (fungus-treated and control without fungus) were fed on a pasture of *Brachiaria decumbens* naturally infected with larvae of bovine trichostrongyles. Animals of the treated group received doses of sodium alginate mycelial pellets orally (1g/10kg body weight, twice a week), for 12 months. Feces samples were collected for egg count (eggs per gram of feces - EPG) and coprocultures during 12 months. There was a significant reduction in EPG (56.7%) and infective larvae (L3) in coprocultures (60.5%) for animals of the treated group in relation to the control group at the end of the study. There was a significant reduction of L3 (64.5%) in herbage samples collected up to 0-20cm from fecal pats and 73.2% in distant samples (20-40cm) between the fungus-treated group and the control group. The treatment with sodium alginate pellets containing the nematode trapping fungus *D. flagrans* reduced trichostrongylid in tropical southeastern Brazil and could be an effective tool for biological control of this parasitic nematode in beef cattle.

Keywords: nematophagous fungus, *Duddingtonia flagrans*, trichostrongyles, cattle, biological control

1. Introduction

Brazil is the world's largest beef exporter with the world's largest commercial cattle herd (USDA, 2011). The beef cattle industry in Brazil is based on grass-fed animals in which the Nellore breed predominates. This constitutes an important advantage for Brazilian beef exportations because the demand for natural beef has been expanding in the world market. In addition, Brazilian packing plants regulate the use of antibiotics, especially ionophores used as growth promoters, and the use of any implant or beta-agonist for cattle is forbidden in Brazil (Millen et al., 2011). But the high incidence of parasites causes the reduction in production and productivity of the herd.

Until recently, the use of anthelmintics has been an effective way to control nematode parasitism, but strains of nematodes resistant to available anthelmintics have emerged. Besides the problem of resistance, there is growing concern worldwide regarding chemical contaminants in beef as well as the risk of environmental contamination (Sutherland and Leathwick, 2011). Thus, the importance of biological control increases, as it is an integrated and sustainable strategy of parasite control (Larsen, 2000). Within the scene of Brazilian cattle, the biological control using natural nematode antagonistic fungi is an interesting alternative for the control of nematode parasitism.

Studies in biological control have aimed to find an isolated fungus able to survive the passage through the gastrointestinal tract, colonize the stool and take effective action on the infective larvae, be easy to produce on a large scale, have the capacity to survive the storage period and not to cause harmful effects on the ecosystem (Waller et al., 1994). Research on nematophagous fungi with potential as biological control candidates have focused on the nematophagous microfungus *Duddingtonia flagrans* (Larsen, 2000). The high potential of *D. flagrans* as a biological agent against free-living stages of bovine trichostrongylid nematodes has been documented in several field studies (Larsen et al., 1995; Dimander et al., 2003; Dias et al., 2007; Boom and Sheath, 2008). However, these studies were conducted with dairy cattle in a season grazing system in temperate climate. To date, there are no studies conducted with beef cattle on extensive year round grazing system and tropical climate.

A major challenge in the implementation of nematophagous fungi for biological control is the development of fungal formulations economically viable and easy to apply. A commonly used alternative for the application of fungi in the biological control is oral administration of chlamydospores mixed into the concentrate and offered as feed to the animals (Larsen, 2000; Knox and Faedo, 2001; Fontenot et al., 2003; Epe et al., 2009). Another alternative for fungi administration has been the sodium alginate formulation (Walker and Connick, 1983; Fravel et al., 1985; Araújo et al., 2004). Sodium alginate formulations containing mycelial mass have been experimentally evaluated against parasitic nematodes of different species in laboratory and field conditions (Dias et al., 2007; Braga et al., 2009). Studies with mycelial mass of nematode-trapping fungus *Arthrobotrys robusta* (Araújo and Sampaio, 2000) and *Monacrosporium thaumasium* (Araújo et al., 2004) encapsulated in sodium alginate and given orally to stabled dairy calves resulted in significant reductions of infective larvae of nematodes, but none of these formulations have been developed using *D. flagrans* for the control of parasitic nematodes of beef cattle in the field. The aim of the present study was to test the effect of the fungus *D. flagrans* (isolated AC001) in sodium alginate pellets for the biological control of parasitic gastrointestinal nematodes on beef cattle reared in extensive systems under the climatic conditions of tropical Brazil.

2. Materials and methods

2.1. Fungal cultures

The isolate (AC001) of *D. flagrans*, a nematode-trapping fungus belonging to the genus *Duddingtonia*, was kept in test tubes containing 2% corn-meal-agar (2% CMA), at 4ºC, in the dark. The isolate was obtained from a Brazilian soil using the soil sprinkling method (Duddington, 1955), modified by Santos et al. (1991). Fungal mycelia were obtained by transferring culture disks (5 mm in diameter) of fungal isolates in 2% CMA to 250 mL Erlenmeyer flasks with 150 mL liquid potato-dextrose medium (Difco), pH 6.5, and incubated under agitation (120 rpm), in the dark at 26ºC, for 10 days. After this period, the mycelium mass was removed and weighed in an analytic scale for the future production of the pellets, which were made in a sodium alginate matrix, according to Walker and Connick (1983) and modified by Lackey et al. (1993).

2.2. In vivo experimental assay

The experiment was conducted at the cattle experimental sector of the Education Center for Forestry and Agricultural Development, Florestal, MG, Brazil, latitude 19°53'22"S, longitude 44°25'57"W, from May 2010 to June 2011.

In the beginning of the experiment, 6–month-old Nellore calves were previously dewormed with 7.5 mL/10kg body weight albendazole 10%. Fifteen days after the anthelmintic treatment, the calves were separated into two groups (fungus-treated and control) of ten animals each.

Calves were allocated to two 15 ha paddocks of *Brachiaria decumbens*, that had been previously grazed by young and adult calves and were naturally infected with bovine trichostrongyle larvae. Then, each animal of the treated group received twice a week 1g pellets/10kg body weight, containing *D. flagrans* mycelial mass (0.2g of fungus/10kg b.w.) combined with 1kg of cattle commercial ration. The treatment was offered during 12 months starting from May 2010. Animals of the control group received 1g pellets/10 kg body weight without fungus.

2.3. Collection and Processing of fecal samples

After the calves had been moved to the paddocks, samples of fresh feces were collected once a week directly from the rectum to determine egg per gram of feces (EPG), according to Gordon and Whitlock (1939). Samples of feces were collected from the animals to observe the growth of the fungi. The feces were incubated in plates containing water agar 2% and 100 L3 recovered from the coprocultures and put into a drying oven at 25°C, for 10 days to confirm the passage and the predatory ability of the fungi through the gastrointestinal tract of cattle and growth in the feces.

Coprocultures were carried out with 20g of feces mixed with ground, moistened and autoclaved industrial vermiculite (NS Barbosa Ind. Com.®) and taken to an oven at 26ºC, for 8 days, to obtain trichostrongyle larvae. Larvae were identified to the genus level as described by Keith (1953). EPG and larvae recovered from coprocultures of animals of both treated and control groups were recorded and percentage of larval reduction was determined according to Mendoza-De-Guives et al. (1999): reduction (%) = mean L3 recovered from control group - mean L3 recovered from treated group / mean L3 recovered from control group x 100.

2.4. Pasture samples

Every 15 days, two herbage samples (0-20 and 20-40 cm away from fecal pats) were collected from both the treated and control groups, from each paddock, in a zigzag pattern from six alternated points, according to Amarante et al (1996). A 500g herbage sample was weighed, and larvae of cattle parasitic nematodes were recovered from there. The pasture samples were put into a drying oven at 100°C, for 3 days, for obtaining the dry matter. The data obtained were transformed in number of larvae per kilogram of dry matter.

2.5. Climate data

Climate data referring to averages of maximum, average and minimum monthly temperatures and monthly rainfall were daily recorded in a meteorological station in the area.

2.6. Treatment of data

The egg count curves (EPG) originated from the coprocultures, number of infective larvae recovered from paddocks (L3), correlation between EPG and recovered L3 were compared over the experimental period. EPG data were transformed into log(x + 1) and then examined by analyses of variance (ANOVA) and Tukey's multiple comparison test with 1% probability. The analyses were performed using the BioEstat 3.0 Software.

3. Results

Figure 1 shows the monthly mean EPG counts. The EPG of animals treated with *D. flagrans* was significantly lower than that of the control group, from August 2010 to June 2011. The total EPG difference between groups was 56.7%. The largest absolute difference (2600 eggs) was found in February 2011. The largest percentage difference was 65.4% found in January 2011. In the last month of the experiment (June 2011), the difference was 53.2%. In the first month of the study (May 2010), the low EPG number was probably due to the previous anthelmintic treatment. The EPG count began growing from October, influenced by temperature and rainfall which were suitable for the nematode life cycle development (Figs. 2 and 3) and peaked in April 2011 at the end of the rainy season (Fig. 1).

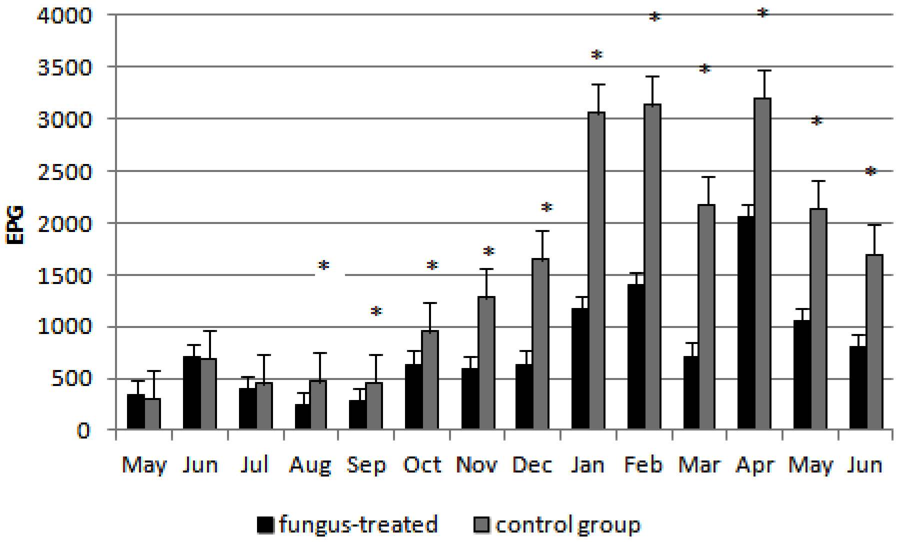


Fig. 1. Monthly means (±SD) of eggs per gram of feces of fungus-treated (black bars) and control animals (gray bars) collected from May 2010 to June 2011, in Florestal, MG, southeastern Brazil. * Significant difference (p<0.01) between the treated and the control group.

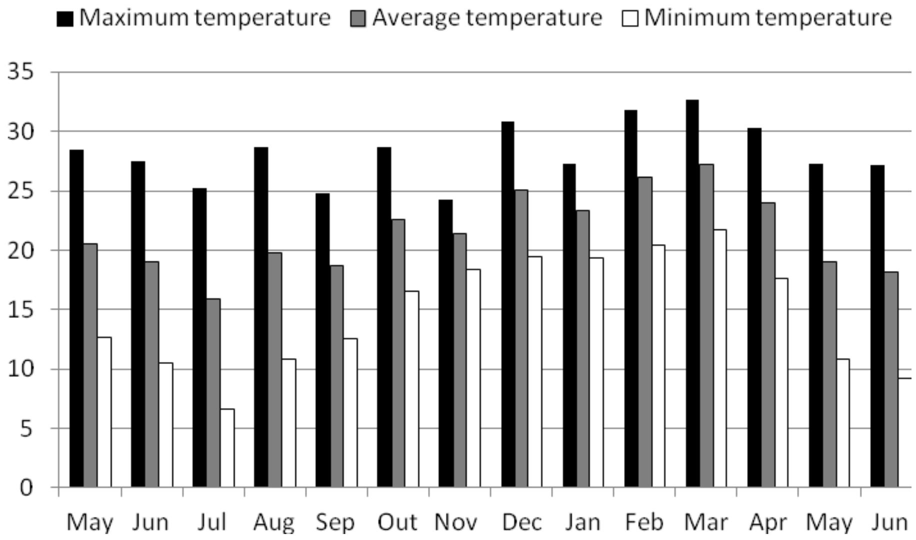


Fig. 2. Maximum, average and minimum monthly temperatures (ºC) recorded from May 2010 to June 2011, Florestal, MG, Brazil.

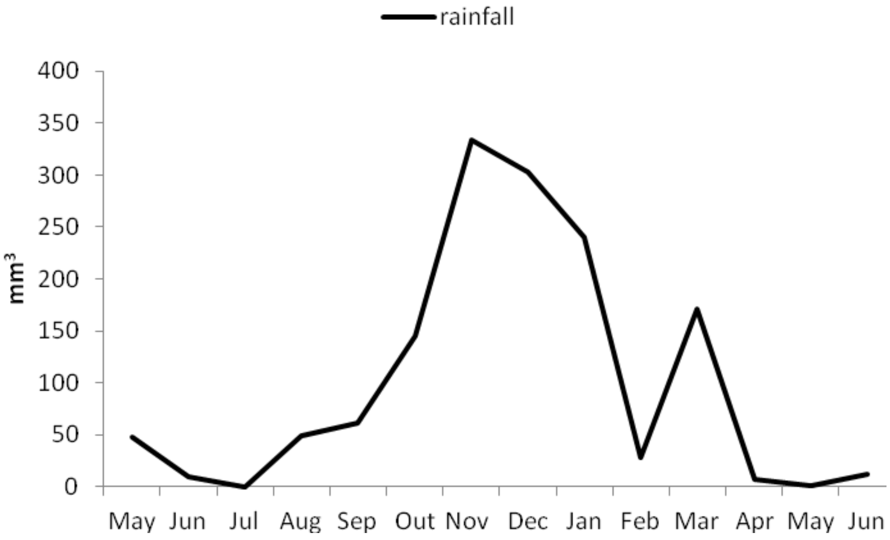


Fig. 3. Monthly rainfall (mm^3) recorded from May 2010 to June 2011, Florestal, MG, Brazil.

Figure 4 shows the coproculture data. There was significant difference between the results of fungus-treated animals and the control group in September, October, November and December 2010, January, March 2011 with larval reduction of 60.5%

after one year of experiment. Larvae recovered from coprocultures belonged mainly to the genera *Cooperia* sp., *Haemonchus* sp. and *Oesophagostomum* sp.. The percentage of nematodes found throughout the study, in the fungus-treated and control groups were respectively: *Cooperia* sp. 48.4% and 44.1%, *Haemonchus* sp. 38.2% and 43.4% and *Oesophagostomum* sp. 12.1% and 10.4%. Other species found in smaller quantities in both groups were *Bunostomum* sp. (0.8 and 1.2%) and *Strongyloides* sp. (0.5 and 0.9%), respectively. No significant difference was found in the proportion of different genera between the treated and control groups.

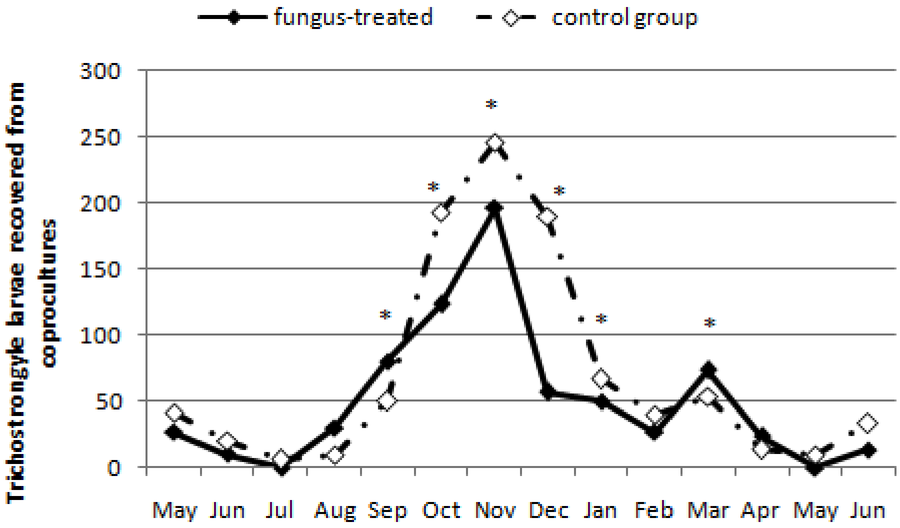
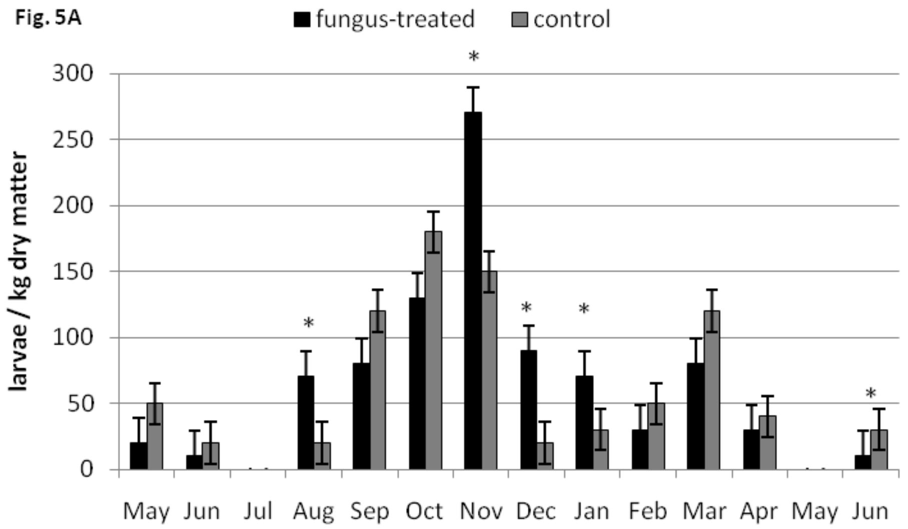


Fig. 4. Mean monthly number of trichostrongylid larvae recovered from coproculture of fungus-treated and control animals collected from May 2010 to June 2011. * Significant difference ($p<0.01$) between the treated and the control group.

Figure 5 shows the number of larvae recovered from paddocks in the distances of 0-20cm (Fig.5A) and 20-40cm (Fig.5B) away from the fecal pats. There was a significant difference of 64.5% for the 0-20 cm samples between the treated group and the control in August, September, December 2010 and January, March 2011. Significant difference of 73.2% was also found for the distance 20-40 cm from the fecal pats between the treated and the control groups in September, October, November, December 2010 and January, February, June 2011. The number of larvae recovered 20 cm away from the fecal pats differed from that found between 20 and 40 cm. Eighty-four percent of the total number of larvae was found within 20 cm from the faecal pat. From September 2010 to January 2011, there was an increase in the number of infective larvae recovered (Figs. 5A and 5B) from the pastures (rainy season). In July 2010 and May 2011, the number of recovered larvae in the pasture was almost zero in both groups. This fact was probably caused by the lack of rain during this period.



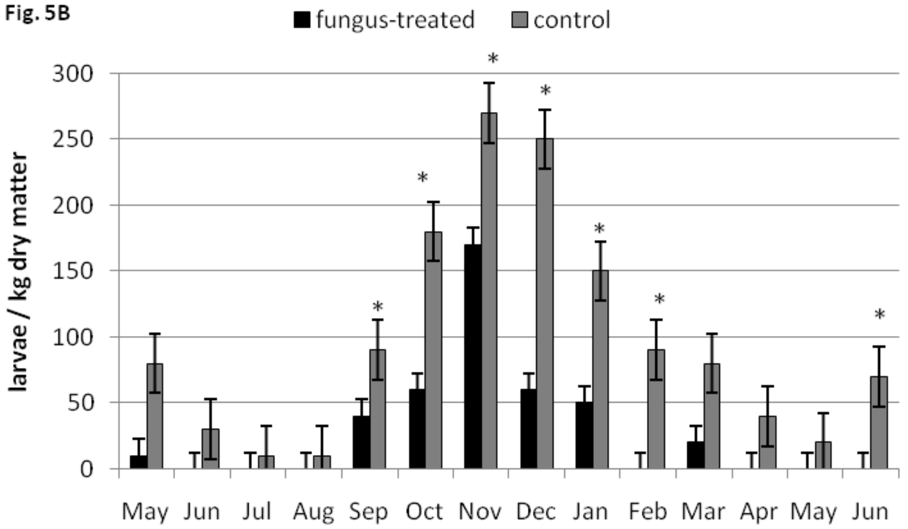


Fig. 5. Monthly counts (±SD) of the number of infective nematode larvae per kilogram of dry matter recovered from pastures of fungus-treated and control animal collected in sampling distances up to 20 cm (A) and 20-40 cm (B) from fecal pats, from May 2010 to June 2011, Florestal, MG, Brazil.

The numbers of larvae of *Cooperia*, *Haemonchus* and *Oesophagostomum* of the treated group recovered from pasture had reductions of 60.5, 53.2 and 47.5%, respectively. During May and June 2010 and April, May and June 2011, the genus *Cooperia* was the most prevalent in the paddocks of the two groups. From July 2010, there was increase in the recovery of *Haemonchus* and *Oesophagostomum* in relation to *Cooperia*. The occurrence of *Oesophagostomum* was higher than *Haemonchus* in

September, December 2010 and February 2011 (during the rainy season) for the two groups.

4. Discussion

According to Raynaud and Gruner (1982) EPG counts allow monitoring the infection levels in animals and of pasture infestation by gastrointestinal nematode parasites. Studies on *D. flagrans* using horses and ruminants recorded average monthly EPG counts lower for treated than for non-treated animals in different locations and climatic conditions: Baudena et al. (2000) for horses in South Louisiana, USA; Knox and Faedo (2001) for sheep in Australia; Fontenot et al. (2003) for ewes in USA; Araújo et al. (2006) for goats in Brazil; Paraud et al. (2007) for goats in France; Braga et al. (2009) for horses in Brazil. The efficacy of *D. flagrans* against gastrointestinal parasites was demonstrated for cattle by Dimander et al. (2003), in which the EPG count decreased approximately 50%. These findings are consistent with results of the present work, confirming the action of the fungus against the infective forms in the fecal environment and consequent decrease in EPG. Araújo et al. (2004) observed a gradual EPG reduction in calves treated with *M. thaumasium* in sodium alginate pellets, obtaining significant reductions from the fourth month after application. The results in Figure 4 indicate that *D. flagrans* acts directly on the infective larvae of trichostrongyles present on the pasture leading to a lower parasitic infection in the animals treated with the fungus (Araújo et al., 2006).

Results of coprocultures revealed the occurrence of *Cooperia*, *Haemonchus* and *Oesophagostomum* over the experimental period (May 2010 - June 2011). This result is in accordance to Dias et al. (2007) that reported *Cooperia* spp. and *Haemonchus* spp. as the most prevalent cattle parasite genera in Brazil, followed by *Oesophagostomum* spp.. The importance of these parasites for the cattle production is directly related with a reduction in weight gain, heavy production losses and the high resistance that these gastrointestinal nematode parasites have developed to anthelmintics (Sutherland and Leathwick, 2011).

Araújo et al. (2004) demonstrated that the fungus *D. flagrans* is not selective for a particular parasites species, which was confirmed in this study. These variations in the proportion of the genera may probably be related to variations in climatic conditions. Regarding the biological control, all genera showed reduction when comparing the treated with the control groups.

Figure 5 shows that the number of larvae found within 0-20 and 20-40 cm away from fecal pats is directly related with the action of nematophagous fungi against the L3 present in pastures, suggesting that *D. flagrans* accounted for the satisfactory reduction of environmental contamination (Araújo et al., 2004). Peña et al. (2002) reported reduction of more than 90% of L3 present in fecal pats of ruminants using *D. flagrans*. Fontenot et al. (2003) also discussed that *D. flagrans* decrease infection forms of gastrointestinal nematode parasites in pasture, avoiding contamination of new animals entering these sites. Clinical parasitism does not occur when biologic control for nematophagous fungi is applied, because a reduced quantity of larvae is available to the animals, sufficient for natural immunity to develop (Araújo et al., 2006).

In the present study, the largest number of L3 was recovered within the distance 0-20 cm away from the fecal pats. This finding corroborates those by Dias et al. (2007), who reported larger numbers of larvae recovered within 0-20 cm from fecal pats, confirming that the few larvae that leave the feces migrate to the herbage beyond 0-20 cm.

Temperature, relative humidity and rainfall promoted the development of free-living stages and migration to the herbage. The optimum temperatures for development of most cattle nematode *Trichostrongylidae* are between 20 and 30°C (O'Connor et al., 2006) and the optimum temperature for growth and predatory activity of nematophagous fungi is between 20 and 30°C (Su et al., 2007). Average temperatures from 16 to 20ºC are suitable for the development of most larvae of cattle parasitic nematodes in pastures. In the climatic conditions of this study, the temperature had no effect in limiting larvae development because the average temperatures observed favor growth and activity of the fungal isolate for almost all the experimental period, except during the months of July 2010 (15.9°C), September 2010 (18.6°C) and June 2011 (18.2°C), whose average temperatures were relatively low.

From May to July 2010 and April to June 2011 (dry season), the rainfall was below 50mm. The minimum rainfall required for larvae development in the environment is 50mm (Boom and Sheath, 2008). However, some studies in Brazil have shown that for some nematode larvae, the moisture content in feces and precipitation from 5 to 7mm is sufficient for their development and subsequent migration to pasture.

During June 2010 and June 2011, the temperature and rainfall were unfavorable for the development of larvae in pastures. Almost no larvae were recovered in the pastures in these months, but even when the rainfall was very low or zero, there was reinfection of the animals, because the larvae were recovered in the feces. These results indicate that the moisture in the fecal pat allows the development of free-living stages of nematodes and that a low rainfall is sufficient for the development and larval migration to the pastures.

Van Dijk et al. (2009) discussed that infection levels are highest during the rainy season and is related to higher humidity, which favors the development of free-living stages of the parasites and migration of L3 from fecal pats to adjacent pastures. The low larvae occurrence on pasture in periods without precipitation and higher scores in the rainy season observed in this study are consistent with these observations. The developmental stage from eggs to L3 occurred throughout the year and migration from fecal pats to pasture is proportional to rainfall. Months with restrictive rainfall hinder self re-infection, thus the number of larvae recovered from pasture was lower. Also, according to Van Dijk et al. (2009), calves might be infected throughout the year in tropical climates, since L3 are always present in the pastures and the grass type can affect larval recovery. Langrova et al. (2003), in central Europe, suggested that L3 respond to rain through dispersion within the vegetation, occurring a moderate correlation between moisture and L3 number in the pasture. Baudena et al. (2000) discussed that the survival of these parasites in the environment is strongly related with temperature and that few larvae would be found in feces in the tropical summer. The author recorded field data in southern Louisiana, a region with subtropical climate in The United States, appearing that there is a larger number of L3 in the pasture in months with mild temperatures. This agrees with the results found in this study, in which the largest number of larvae recovered in pastures was found during months of mild temperatures (Fig. 5).

It is likely that the reduction in the EPG in the treated group was caused by the application of the *D. flagrans* nematophagous fungus, that when acting on the free living forms of the nematodes, decreased pasture contamination and consequently of risk of reinfection in the paddock where the animals were treated with the fungus. These results are similar to those reported by Larsen et al. (1995) and Dimander et al. (2003), who studied the effect of the application of the *D. flagrans* on gastrointestinal nematodes of cattle.

5. Conclusion

Treatment of beef cattle with pellets containing mycelial mass of the nematophagous fungus *D. flagrans* can be effective to control trichostrongyles in tropical southeastern

Brazil. This is the first study of biological control using an isolate of *D. flagrans* in tropical climate and beef cattle. Our results showed that that treatment of beef calves with sodium alginate pellets containing mycelial mass administered twice a week reduced the pasture infestation by infective larvae of nematodes during the rainy and dry seasons in the southeastern Brazil. The findings of this study are encouraging for the use of nematophagous fungi in the biological control of the gastrointestinal nematode of beef cattle in natural conditions.

Acknowledgements

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CAPÍTULO 2 - *Journal of Helminthology*

An isolate of the nematophagous fungus *Monacrosporium thaumasium* for the control of cattle trichostrongyles in southeastern Brazil

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Abstract

A mycelial formulation in sodium alginate pellets of the nematophagous fungus *Monacrosporium thaumasium* (isolate NF34A) was assessed in the biologic control of beef cattle trichostrongyles in tropical Brazil. Two groups (fungus-treated and control without fungus) of ten six-month-old male Nellore calves were fed on a pasture of *Brachiaria decumbens* naturally infected with larvae of cattle trichostrongyles. The treated group received doses of sodium alginate mycelial pellets orally (1g pellets (0.2g of fungus)/10kg live weight) twice a week for 12 months. There was a significant reduction ($p<0.01$) in the number of eggs per gram of faeces (47.8%) and coprocultures (50.2%) of the treated group in relation to the control group at the end of the study. There was a 47.3% reduction in herbage samples collected up to 0-20 cm between the fungus-treated and control groups and a 58% reduction in the sampling distance 20-40 cm from faecal pats ($p<0.01$). The treatment with sodium alginate pellets containing the nematode trapping fungus *M. thaumasium* reduced trichostrongyles in tropical southeastern Brazil and could be effective tools for the biological control of this parasitic nematode in beef cattle. However in such a tropical climate with low rainfall the fungal viability can be reduced.

Keywords: nematophagous fungus, *Monacrosporium thaumasium*, trichostrongyles, beef cattle, biological control

Introduction

Gastrointestinal helminthiasis can cause heavy production losses in dairy and beef cattle and has become an important factor in the world economy. The productivity indices in Brazil are considered low, mainly because of the seasonal fluctuation in pasture production, especially in the dry season, and the parasitic diseases (Souza *et al*., 2008).

Fiel *et al*. (2006) reported that in countries of Oceania, Europe and South America, such as Brazil, Argentina and Uruguay, anthelmintic resistance in nematode parasites has become serious problem in cattle production systems. In Brazil, Pinheiro & Echevarria (1990) reported for the first time resistance of *H. contortus* to albendazole and oxfendazole in cattle. Subsequently, several other reports described helminth resistance in cattle in Brazil including the resistance of *Trichostrongylus* spp and *Ostertagia* spp to levamisole, *Haemonchus* spp and *Cooperia* spp to ivermectin and *Cooperia* spp to albendazole sulfoxide (Souza *et al*., 2008).

Non-chemotherapeutic approaches for the control of nematode parasites of livestock are no longer of academic interest alone and alternatives or adjuncts to anthelminthic drugs are now considered to be necessary (Araújo *et al*., 2004). Thus, biological control with nematophagous fungi in cattle can be a viable alternative, acting in reducing environmental contamination by free-living stages of these parasites, reducing the frequency of chemicals treatments and thus reducing the dependence on anthelmintics.

Species of *Monacrosporium* (Hyphomycetales) can control phytonematodes, free-living nematodes and parasitic nematodes of cattle (Araújo *et al*., 1992; Gomes *et al*., 1999; Araújo *et al*., 2004). However, there are no reports of studies with beef cattle in tropical climates either in rainy or dry season.

The objective of the present study was to assess the viability of a formulation with the fungus *Monacrosporium thaumasium* in the biological control of beef cattle gastrointestinal nematode parasites in tropical Brazil.

Materials and methods

Fungal cultures

An isolate of *M. thaumasium* (NF34A), a nematode-trapping fungus belonging to the genus *Monacrosporium*, was maintained in test tubes containing 2% corn-meal-agar

(2% CMA) in the dark, at 4ºC. Fungal mycelia were obtained by transferring culture disks (5 mm in diameter) of fungal isolates in 2% CMA to 250 mL Erlenmeyer flasks with 150 mL liquid potato-dextrose medium (Difco, Brazil), pH 6.5. The cultures were incubated under agitation (120 rpm), in the dark at 26ºC, for 10 days. Mycelia were then removed and pellets were prepared by using sodium alginate as described by Walker & Connick (1983) and modified by Lackey *et al*. (1993).

In vivo experimental assay

The study was performed on the cattle experimental sector of the Education Center for Forestry and Agricultural Development, Florestal, state of Minas Gerais, Brazil, latitude 19°53'22"S, longitude 44°25'57"W, from May 2010 to June 2011.

Twenty six-month old Nellore calves were previously treated with 7.5 mL/10kg body weight albendazole 10%. Fifteen days after the anthelmintic treatment, the calves were separated into two groups (fungus-treated and control) of ten animals each. Calves were allocated to two 15ha paddocks of *Brachiaria decumbens* that had been previously grazed by young and adult calves and were naturally infected with cattle trichostrongylid larvae. Each animal of the treated group received twice a week 1g pellets/10 kg body weight, containing *M. thaumasium* mycelial mass combined with 1kg of cattle commercial ration. The treatment was offered during 12 months, starting from May 2010. The control group received 1g pellets/10 kg body weight without fungus.

Collection and examination of faecal and herbage samples

Samples of fresh faeces were collected once a week directly from the rectum to determine egg per gram of faeces (EPG), according to Gordon & Whitlock (1939). Samples of faeces were collected from the animals to observe fungal growth. The faeces samples were plated on water agar 2% with 100 cattle trichostrongylid larvae and incubated at 25ºC, for 10 days. Twenty grams of faeces were mixed with soil and moist autoclaved industrial vermiculite and incubated at 26ºC for 8 days to obtain trichostrongylid larvae. Larvae were identified to the genus level as described by Keith (1953). EPG and larvae recovered from coprocultures of animals of both treated and control groups were recorded and percentage of larval reduction was determined according to Mendoza-De-Guives *et al*. (1999): reduction (%) = mean L3 recovered from control group - mean L3 recovered from treated group / mean L3 recovered from control group x 100.

Every 15 days, two herbage samples were collected from each paddock, in a W pattern from alternated points, 0-20 and 20-40 cm away from faecal pats, according to Amarante *et al.* (1996). A 500g herbage sample was weighed, and parasitic nematode larvae were recovered following the procedure of Lima (1989). The 500g samples were incubated in a drying oven at 100ºC, for 3 days, to determine dry matter. Data were transformed into larvae per kg of dry matter. Climate data referring to averages of maximum, average and minimum monthly temperatures and monthly rainfall were daily recorded by a meteorological station in the area.

Data analysis

Egg count curves originated from the coprocultures, number of infective larvae recovered from paddocks (L3), correlation between EPG and recovered L3 were compared over the experimental period. Data were examined by Student T test at 1% probability level.

Results

EPG counts in calves treated with *M. thaumasium* was significantly lower ($p<0.01$) than that of the control group between August 2010 and June 2011 (Fig. 1). At the end of the study, the total EPG difference between groups was 47.8%. There was no significant difference between the treated group and the control during the three first months of the experiment. In the first month of the study (May 2010), the EPG count was affected by the previous anthelmintic treatment. The largest absolute difference (1900 eggs) and the largest percentage difference (64%) were found in January 2011. In the last month of the experiment (June 2011), the difference was 32.3%. The EPG count started rising from October, influenced by temperature and rainfall (Fig. 4). The EPG counts peaked in end of rainy season.

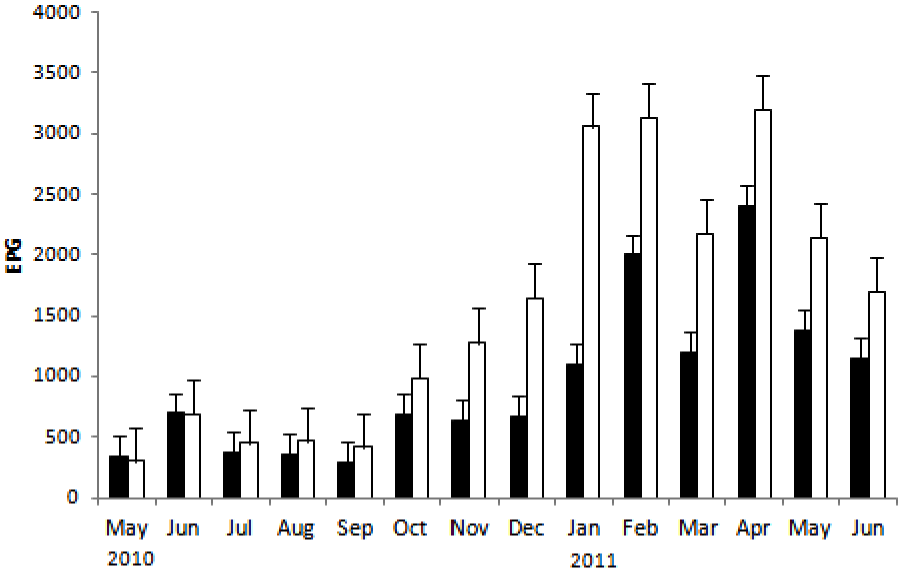

Fig. 1. Mean monthly numbers (+/-SD) of eggs per gram of faeces (EPG) of fungus-treated (black square) and control (open square) calves from May 2010 to June 2011, in Florestal, Minas Gerais, southeastern Brazil; $p<0.01$ between treated and control groups except in May-July 2010 and June 2011.

There was significant difference between the coproculture data of fungus-treated animals and the control group in October, November, December 2010 and January 2011, with larval reduction of 50.2% after one year of experiment (Fig. 2). The coprocultures showed that *Cooperia* sp. was the most prevailing gastrointestinal parasitic nematode in both groups, comprising 53.1% and 44.1% of the parasitic population in animals of the groups A and B respectively, while *Haemonchus* comprised 34.7% and 43.4% and *Oesophagostomum* 10.9% and 10.4% of the parasitic population in the groups A and B, respectively. Other species found in smaller quantities in the groups A and B were *Bunostomum* sp. (0.6% and 1.2%) and *Strongyloides* sp. (0.7% and 0.9%), respectively. No difference ($p>0.01$) was found in the proportion of different genera between the animals of the two groups.

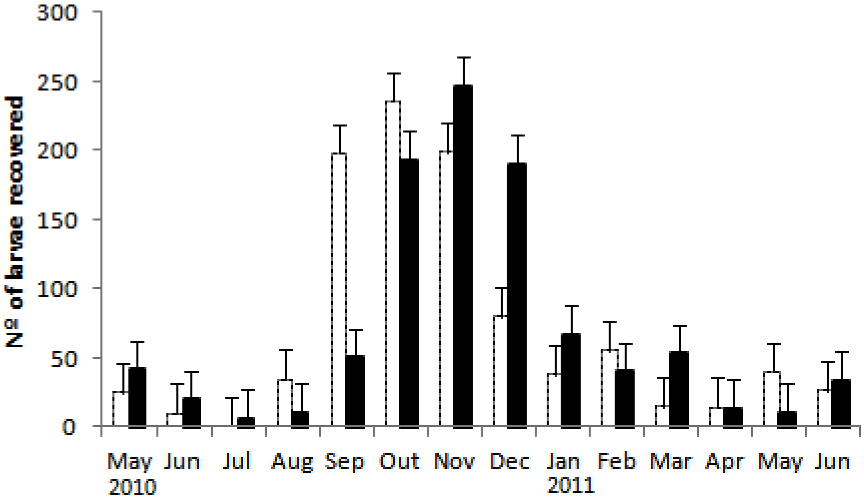

Fig. 2. Mean monthly numbers (+/-SD) of trichostrongylid larvae recovered from coproculture of fungus-treated (□) and control (■) calves from May 2010 to June 2011; error bars indicate p< 0.01 in October, November, December 2010 and January 2011.

There were statistical differences in the number of L3 recovered from paddocks in the distances (0-20cm) and (20-40cm) from the faecal pats (Fig. 3). At the end of the experiment, there was a significant difference (p<0.01) of 47.3% for the 0-20 cm samples and a significant difference (p<0.01) of 58% for the 20-40 cm samples between the treated group and the control. From November 2010 to January 2011, there were significant differences between the treated and control group at both distances (0-20 cm) and (20-40 cm). The number of larvae recovered up to 20 cm from the faecal pats differed from that found between 20 and 40 cm (p<0.01). A total of 91% of the larvae were found up to 20 cm from the faecal pats and only 9% from 20 to 40 cm. In the control group, the highest percentage of L3 was also found up to 20 cm from the faecal pat (73%), but there was a greater amount of L3 recovered from 20 to 40cm (27%) compared with the treated group. The larval numbers of *Cooperia*, *Haemonchus* and *Oesophagostomum* of the treated group recovered from the pasture (per Kg dry matter) had reductions of 33.6%, 26.6% and 57.5%, respectively.

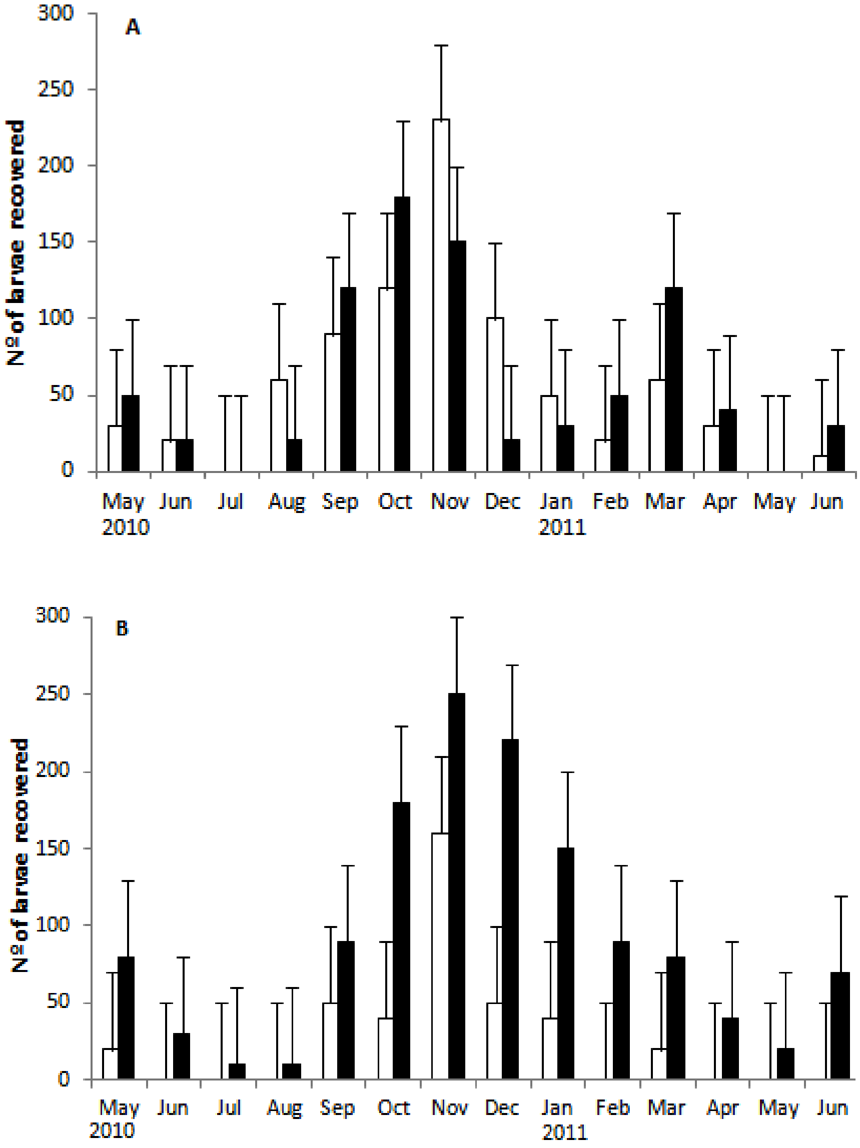

Fig. 3. Mean monthly numbers (+/-SD) of number of infective nematode larvae per kilogram of dry matter recovered from pastures of fungus-treated calves A) fungus-treated (□) and control (■) collected up to 20cm from faecal pats and B) fungus-treated (□) and control (■) collected up to 20-40cm from faecal pats, May 2010 to June 2011, Florestal, Minas Gerais, Brazil; p<0.01 between treated and control groups in November, December 2010 and January 2011.

The monthly average temperature ranged from 15.9°C (July 2010) to 27.2°C (March 2011) (Fig. 4).

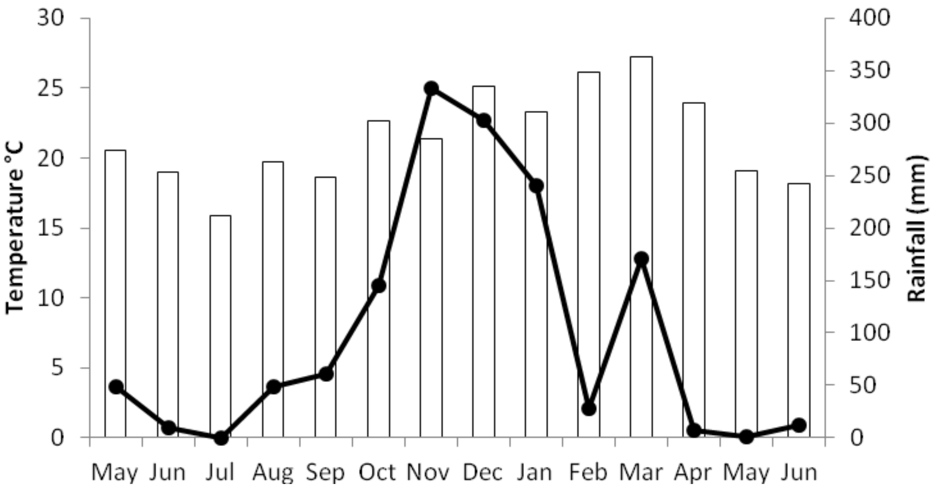

Fig. 4. Mean values of monthly temperatures (□) in °C and rainfall in mm3 (--•--) recorded from May 2010 to June 2011, Florestal, Minas Gerais, Brazil.

Discussion

Species of the genus *Monacrosporium* such as *M. appendiculatum, M. ellipsosporum, M. sinense* and *M. thaumasium* have proved to be effective in the control of gastrointestinal nematodiasis of different animal species (Assis & Araújo, 2003; Melo *et al*., 2003; Araújo *et al*., 2006; Campos, 2006; Araújo *et al*., 2007). These reports corroborate the findings of the present study, in which decrease in EPG count was found in animals of the group treated with *M. thaumasium*.

Araújo *et al*. (2004) reported a gradual EPG reduction in calves treated with the same formulation of *M. thaumasium* isolate in sodium alginate pellets used in this study, obtaining significant reductions from the fourth month after the application. The authors reported that at the end of the six-month study period (September 2000 to August 2001) the difference between the EPG count of the treated group and the control group was almost 100%. This result differs from the findings of the present work, in which the EPG decrease was 47.8%. This difference was probably due to different climatic conditions between the study sites. In the region where Araújo *et al*. (2004) conducted their study, the seasons of higher rainfall coincide with the best pasture conditions, however, the conditions of our study were of low rainfall and high average temperatures.

The coproculture data (Fig. 2) indicate that *M. thaumasium* acted directly on the trichostrongylid infective larvae present on the pasture, leading to a lowering of parasitic infection in the animals treated with the fungus. The coprocultures showed the occurrence of different genera of Trichostrongylid. Dias *et al*. (2007) reported that

Cooperia spp. and *Haemonchus* spp. are the most prevalent cattle parasite genera in Brazil, followed by *Oesophagostomum* spp.. *Cooperia*, *Haemonchus* and *Oesophagostomum* were the most common genera found in the coprocultures and pasture over the experimental period (May 2010 - June 2011). The importance of these parasites for the cattle production is directly related with a reduction in weight gain, heavy production losses and the high resistance that these gastrointestinal nematode parasites have developed to routine anthelmintics (Araújo *et al*, 2004).

During May and June 2010 and April, May and June 2011, the genus *Cooperia* was the most prevalent in the coprocultures of the two groups. From July 2010, there was increase in the recovery of *Haemonchus* and *Oesophagostomum* in relation to *Cooperia*. The occurrence of *Oesophagostomum* was higher than *Haemonchus* in September, December 2010 and February 2011 (during the rainy season) in the two groups. These variations in the proportion of the genera are related to variations in climatic conditions (rainfall and temperature). With respect to the biological control, there was reduction of infective larvae of all genera when comparing the treated group with the control.

The number of larvae found within 0-20 and 20-40 cm away from faecal pats is directly related with the action of nematophagous fungi against the L3 present in pastures, suggesting that *M. thaumasium* accounted for the satisfactory reduction of environmental contamination (Fig. 3). According to Araújo *et al.* (2004), the oral administration of *M. thaumasium* pellets resulted in the control of pasture infestation close to 100% during February-August 2001. In the present study, the reductions in L3 recovered from pastures were 47.3% (0-20cm) and 58% (20-40 cm). The difference in these results may be explained by the low rainfall and higher temperatures prevalent in the region of our study, which may have -reduced the efficiency of the fungus. The largest number of infective larvae was recovered within the distance 0-20 cm away from the faecal pats. This finding corroborates the results of Dias *et al.* (2007), who reported larger numbers of larvae recovered within 0-20 cm from faecal pats, confirming that the few larvae that leave the faeces migrate to the herbage further than 0-20 cm. According to Saueressig (1980), if the environmental conditions are not favorable to translation (when eggs present in the faeces reach the infective stage on herbage available to the grazing animal), the larvae can survive in the pat, especially when the pat remains intact. Temperature, relative humidity and rainfall promoted the development of free-living stages and migration to the herbage (Fig. 4). The optimum temperature range for the development of most *Trichostrongylidae* of cattle is between 20°C and 30°C (O'Connor, 2006) and the optimum temperature for growth and predatory activity of nematophagous fungi is between 20°C and 30°C (Su *et al.*, 2007). Average temperatures from 16ºC to 20ºC are suitable for the development of most larvae of cattle parasitic nematodes in pastures. In the climatic conditions of this study, the temperature had no effect in limiting larvae development because the average temperatures favored growth and action of the fungal isolate over almost all the experimental period, except during the months of July 2010 (15.9°C), September 2010 (18.6°C) and June 2011 (18.2°C), in which average temperatures were relatively low.

From May to July 2010 and April to June 2011 (dry season), the rainfall was below 50mm. The minimum rainfall required for larvae development in the environment is 50mm (Boom & Sheath, 2008). However, some studies in Brazil have shown that for some nematode larvae, the moisture content in faeces and precipitation from 5 to 7mm would be sufficient for their development and subsequent migration to pasture (Lima, 1989).

During June 2010 and June 2011, both temperature and rainfall were unfavorable for the development of eggs and larvae in pastures. Almost no larvae were recovered from the paddocks in these months, but even when the rainfall was very low or zero, there was reinfection of the animals because the larvae were recovered in the faeces. These results indicate that the moisture in the faecal pat allows the development of free-living stages of nematodes and that low rainfall is sufficient for the development and larval migration to the pastures. Under the conditions of this study, it can be considered that the availability of water would be the limiting condition for the occurrence of translation, since the temperatures recorded in the period are notoriously favorable to it. Our data clearly show the close relationship between the migration of infective larvae present in the stool to the pasture and rainfall.Van Dijk *et al.* (2009) discussed that infection levels are highest during the rainy season and related to higher humidity, which favors the development of free-living stages of the parasites and migration of infective larvae from faecal pats to adjacent herbage.

The low occurrence of larvae on pasture in periods without precipitation and higher scores in the rainy season observed in this study is consistent with these observations. The developmental stage from eggs to infective larvae occurred throughout the year and migration from faecal pats to pasture is proportional to rainfall. Months with restrictive rainfall hinder self re-infection, thus the number of larvae recovered from pasture was lower.

Van Dijk *et al.* (2009) also argued that calves might be infected throughout the year in tropical climates, since L3 are always present in the pastures and the grass type can affect larval recovery. Langrova *et al.* (2003), in central Europe, suggested that L3 respond to rain through dispersion within the vegetation, with a moderate correlation between moisture and L3 number in the pasture. Baudena *et al.* (2000) discussed that the survival of these parasites in the environment is strongly related with temperature and that few larvae would be found in faeces in the tropical summer. They recorded field data in southern Louisiana, a region with subtropical climate in the United States of America, appearing that there are a larger number of infective larvae in the pasture in months with mild temperatures. This agrees with the results found in this study, in which the largest number of larvae recovered in pastures was found during months of mild temperatures (Fig. 3).

Treatment of beef cattle with pellets containing mycelial mass of the nematophagous fungus *M. thaumasium* was effective to control trichostrongyles in tropical southeastern Brazil. This is the first report of biological control using *M. thaumasium* to control beef cattle trichostrongyles in tropical climate. The treatment of beef calves with sodium alginate pellets containing mycelial mass administered twice a week reduced pasture infestation by infective larvae of nematodes during the rainy and dry seasons in southeastern Brazil.

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CAPÍTULO 3 - *Veterinary Parasitology* 193 (2013) 134-140

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Comparison between the action of nematode predatory fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* in the biological control of bovine gastrointestinal nematodiasis in tropical southeastern Brazil

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Abstract

Sodium alginate pellets of the nematode predatory fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* were evaluated in the biological control of bovine gastrointestinal nematodiasis. Three groups (A, B and C) of ten six month old male Nelore bulls were kept in paddocks of *Brachiaria decumbens* for 12 months. Each animal of group A received 1g/10kg of body weight (b.w.) of pellets of *D. flagrans* (0.2g of fungus/10kg b.w.) and of group B, 1g/10kg of b.w. of pellets of *M. thaumasium* (0.2g of fungus/10kg b.w.), twice a week, for 12 months. Animals of the group control received no fungus. The monthly averages of egg count per gram of feces of the animals of groups A and B were 56.67% and 47.8% smaller, than the animals of group C ($p<0.05$), respectively. Treatment of bulls with pellets containing the nematophagous fungi *D. flagrans* and *M. thaumasium* can be used as an alternative treatment of bovine gastrointestinal nematodiasis, however, *D. flagrans* was more efficient than *M. thaumasium* for the biological control in the environmental conditions of the present study.

KEYWORDS: Cattle-Nematoda; Biological Control; *Duddingtonia flagrans*; *Monacrosporium thaumasium;* bovine

1. INTRODUCTION

Research on the application of the nematode-trapping fungi *D. flagrans* (Larsen et al. 1995; Sarkunas et al., 2000; Dias et al., 2007) and *M. thaumasium* (Alves et al., 2004; Araújo et al., 2004) for treating bovine gastrointestinal nematodiasis has demonstrated their potential as biological control agents against the free-living stages of parasitic nematodes in livestock under experimental and natural conditions. However, most studies of biological control in cattle have been conducted in temperate regions (Larsen et al. 1995; Sarkunas et al., 2000) with dairy cattle that is usually kept in pasture only in the grazing season. There are no reports of previous studies evaluating biological control with *D. flagrans* or *M. thaumasium* in extensive systems of beef cattle and, due to weather conditions, maintained on pasture throughout the year. Sodium alginate based formulations have been evaluated experimentally in the control of animal parasitic nematodes by some research groups. Such formulations have provided good results under laboratory and field conditions (Araújo et al., 2000). Various nematophagous fungi have been successfully used in formulations based on sodium alginate against gastrointestinal helminthes; however, few studies have been conducted with the fungi *M. thaumasium* (Alves et al., 2004; Silva et al., 2009) or *D. flagrans* (Campos et al., 2007; Silva et al., 2009) using these formulations and under natural conditions.The objective of this study was to evaluate the effectiveness of the nematophagous fungi *D. flagrans* and *M. thaumasium* in sodium alginate pellets in the biological control of gastrointestinal nematodiasis in beef cattle raised in the field, in a tropical climate.

2. MATERIALS AND METHODS

2.1. Area of study

The experiment was carried out in the experimental farm of the Federal University of Viçosa, located in the municipality of Florestal, state of Minas Gerais, southeast region of Brazil, 19°53'22"south latitude and 44°25'57"west longitude, from May 2010 to June 2011.

The paddock’s topography is flat to hilly (29% flat, 54% undulating and 17% hilly), with an average altitude of 750 m and native vegetation of forest-cerrado transition zone. The climate is tropical with a dry season (Rating Köppen-Geiger climate: Aw), with annual average maximum temperature of 28 ° C and minimum of 13.9 ° C.

2.2. Fungal cultures

Two isolates of the predatory fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were kept in test tubes containing corn meal agar 2%. (2% CMA, Difco$^{®}$, USA), at 4°C in the dark. These isolates came from a Brazilian soil and belonged to the mycology collection of the Federal University of Viçosa, Brazil. To induce the formation of the fungal mycelium, culture discs of 5mm in diameter were transferred to 250mL Erlenmeyer flasks containing 150mL of potato dextrose (Difco$^{®}$, USA) liquid medium, pH 6.5, under the agitation of 120 rpm, in the dark, 26°C, for 10 days. After this period, the mycelia were harvested with a platinum loop, and weighed in an analytic scale for the future production of the pellets, which were made in a sodium alginate matrix, according to Walker and Connick (1983) and modified by Lackey et al. (1993).

2.3. Experimental animals

In the beginning of the experiment, thirty 6-month-old male Nelore bulls, with average body weight of 120 kg were previously treated with 10% albendazole (Mogivet Lab$^{®}$, Brazil), at an oral dose of 7.5ml/10kg of b.w. Fifteen days after the antihelminthic treatment, the animals were separated into three groups (A, B, and C) of 10 bulls each, based on the average weight. The bulls were allocated to three 15.0 ha paddocks of *Brachiaria decumbens*, naturally infested with gastrointestinal parasite helminths, due to the previous grazing by young and adult animals. Each group was allocated to only one paddock without rotational grazing between the groups during the experimental period. Each animal of group A received 1g of pellets (0.2g of fungal mycelium) for each 10kg of b.w. containing the fungus *D. flagrans* (AC 001) while, in group B, each animal received 1g of pellets (0.2g of fungal mycelium) for each 10kg of b.w. containing the fungus *M. thaumasium* (NF34a). The animals of group C received 1g of fungus-free pellets for each 10kg of b.w. All the animals received the pellets orally, twice a week, mixed in concentrated and balanced ration provided for beef cattle (13% of total protein - Federal University of Viçosa), and water *ad libitum* during 12 months, starting from May 2010.

After the introduction of the bulls into the paddocks, samples of feces were collected, once a week, directly from the rectum, to determine the number of nematode eggs per gram of feces (EPG), according to Gordon and Whitlock (1939). Every day, meteorological data were recorded in a specialized station in the region, referring to the averages of the maximum, average, and minimum monthly temperatures and rainfall.

Fecal samples were collected from the animals to observe the growth of the fungi, once a week, two days after treatment with fungi. The feces were incubated in plates containing water agar 2% and 100 L3 recovered from the coprocultures and put into a drying oven at 25°C, for 10 days to confirm the passage and the predatory ability of the fungi through the gastrointestinal tract of cattle and growth in the feces. Simultaneously to the EPG exam, coprocultures were carried out, for each animal, according to the methodology described by Roberts et al. (1952). The identification of the infectant larvae in the coprocultures was performed according to Keith (1953). Egg count per gram of feces (EPG) and larvae recovered from coprocultures of animals from both treated and control groups were recorded, and percentage of larval reduction was determined according to Mendoza-De-Guives et al. (1999): Reduction (%) = Mean L3 recovered from control group - Mean L3 recovered from treated group x 100/ Mean L3 recovered from control group.

Every 15 days, herbage samples were collected in each paddock of groups, in a zigzag pattern from alternated points, 20 cm and 20–40 cm far from the fecal pats, according to Amarante et al. (1996). Then, a 500g herbage sample was weighed, and parasitic nematode larvae were recovered following the procedure of Lima (1989). The samples were incubated in a drying oven at 100°C for 3 days to determine the dry matter content. Data were transformed into larvae per kg of dry matter.

The EPG, number of infective larvae (L3) recovered from the feces and paddocks, and correlation between EPG and recovered L3 were compared over the experimental period. The weight of the animals was compared during the months of the experiment, starting from June 2010. EPG data were transformed into log(x+1) prior to analysis. Data was examined by analyses of variance and Tukey’s multiple comparison test with 1% probability. Correlation analyses were performed by Pearson’s correlation test ($p<0.001$).

3. RESULTS

The monthly average values of EPG counts are shown in Fig. 1. In the first three months of the experiment (May, June and July 2010) no statistical difference was observed (p>0.05) between the groups treated with fungi (A and B) and the control group (C). In the first month of the study (May 2010), the low EPG number was probably due to the previous anthelmintic treatment. EPG values increased during the rainy season (October from March) and peaked in April 2011 (2060, 2400, 3200 eggs in groups A, B and C, respectively) at the end of the rainy season (Fig. 1).

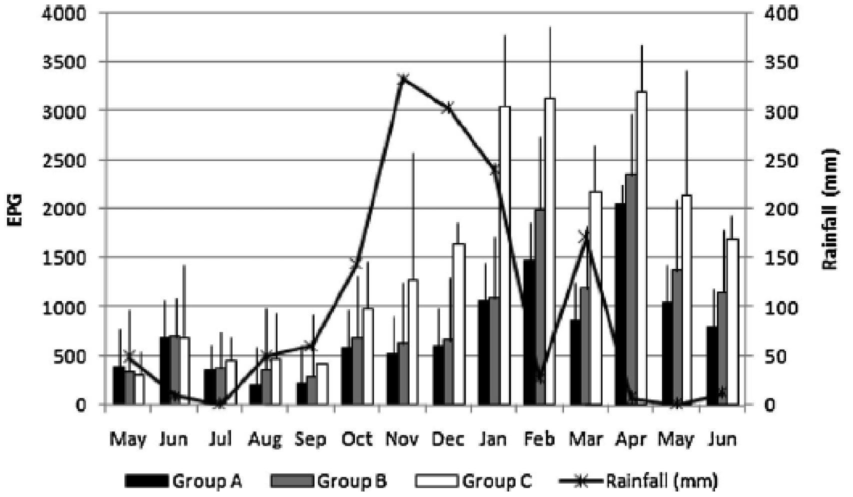


Fig. 1 Monthly averages and standard deviations of the countings of eggs per gram of feces (EPG) of the animals in the groups treated with the nematophagous fungi *D. flagrans* and *M. thaumasium* (1g of fungus/10kg of body weight) and the control group, collected from May 2010 to June 2011, Florestal, Minas Gerais, Brazil.

The EPG counts of animals treated with *D. flagrans* (Group A) and *M. thaumasium* (Group B) were significantly lower than those of the control group from August 2010 to June 2011 (p>0.05). However, the EPG counts of animals treated with *D. flagrans* were significantly lower than those of the animals treated with *M. thaumasium* on August 2010 and February, March, April, May, June 2011. There was no difference between the EPG of the two groups treated with fungus in September, October, November, December 2010 and January 2011.

The monthly average of the EPG of the animals in the group treated with pellets containing the fungus *D. flagrans* was 56.7% lower than that of the animals in the control group at the end of the experiment. For the animals of group B treated with pellets containing the fungus *M. thaumasium*, the reduction observed was of 47.8% in comparison with the animals in the control group. EPG decreased significantly more in *D. flagrans* than in *M. thaumasium* at the end of the experiment.

Figure 2 shows the maximum, average and minimum temperature and monthly rainfall.

Table 1 shows the percentage values corresponding to the infectant larvae (L3) recovered from the coprocultures.

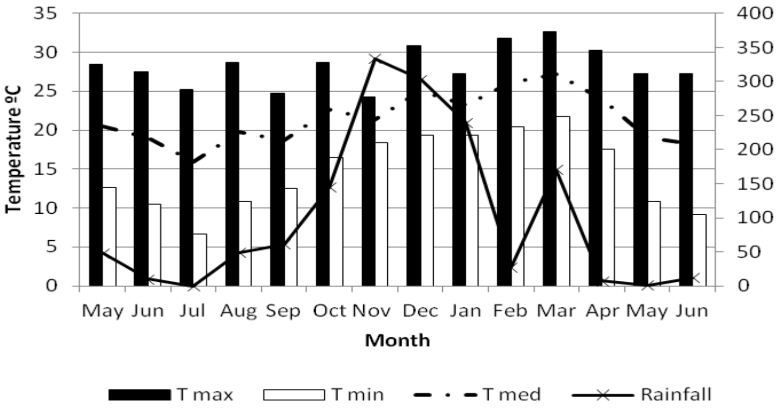


Fig. 2. Averages of maximum, average and minimum monthly temperatures (ºC) and monthly rainfall (mm^3) recorded from May 2010 to June 2011, Florestal, MG, Brazil.

Tab. 1. Percentage values corresponding to the infectant larvae (L3) recovered from the coprocultures of the groups treated with the nematophagous fungi *D. flagrans* (Group A) and *M. thaumasium* (Group B) (1g of fungus/10kg of body weight) and control group (Group C), collected from May 2010 to June 2011, in Florestal, Minas Gerais, Brazil.

	Group A			**Group B**			**Group C**		
	Coop	**Haem**	**Oeso**	**Coop**	**Haem**	**Oeso**	**Coop**	**Haem**	**Oeso**
May	45	55	0	39	61	0	61	39	0
Jun	29	71	0	21	71	4	75	25	0
Jul	62	35	3	72	24	0	41	53	6
Aug	68	27	5	74	18	3	29	63	8
Sep	75	11	14	82	10	2	22	68	10
Oct	65	25	10	75	8	17	22	60	18
Nov	76	11	9	77	9	14	17	56	7
Dec	36	8	56	61	29	8	18	52	25
Jan	55	33	12	41	10	49	40	41	19
Feb	44	25	31	61	27	14	35	29	32
Mar	50	34	6	76	5	20	46	49	5
Apr	32	54	14	14	77	9	69	24	7
May	26	64	10	28	67	5	63	28	9
Jun	15	82	0	23	70	7	79	21	0
Average	48,4	38,2	12,1	53,1	34,7	10,9	44,1	43,4	10,4
S.D.	18,5	22,5	14,4	23,6	26,8	12,2	21,0	15,4	9,3

The coprocultures showed that *Cooperia* sp. was the most prevailing gastrointestinal parasitic nematode in the experiment in all the groups, with percentages of 48.4%, 53.1%, and 44.1% for the animals of the groups A, B, and C, respectively. *Haemonchus* was 38.2, 34.7 and 43.4% and *Oesophagostomum* was 12.1, 10.9 and 10.4%. Other species found in smaller quantities in the groups A, B and C were *Bunostomum* sp. (0.8, 0.6 and 1.2%) and *Strongyloides* sp. (0.5, 0.7 and 0.9%), respectively. No difference (p>0.01) was found in the proportion of different genera between the animals of the three groups.

Table 2 shows the absolute values of L3 per kg of dry matter obtained from pastures grazed by the three groups of cattle.

Tab. 2. Values of L3 per kg of dry matter obtained from pastures grazed by the groups treated with the nematophagous fungi *D. flagrans* (Group A) and *M. thaumasium* (Group B) and control group (Group C).

	Group A			**Group B**			**Group C**		
	Coop	**Haem**	**Oeso**	**Coop**	**Haem**	**Oeso**	**Coop**	**Haem**	**Oeso**
	4	5	0	9	6	0	7	3	0
	3	7	0	5	4	0	7	5	0
	1	1	0	2	1	0	4	3	0
	2	1	0	2	2	0	3	2	0
	2	7	0	2	6	0	13	21	3
	6	8	2	10	8	2	15	18	2
	3	8	5	5	23	7	12	12	16
	15	9	5	21	17	8	28	24	15
	10	3	2	19	10	4	22	11	12
	4	2	1	6	9	1	8	7	3
	11	4	4	21	8	2	26	19	5
	3	5	1	4	2	0	6	2	0
	1	1	10	2	0	0	7	2	0
	1	0	0	3	0	0	9	2	0
e	4,7	4,4	2,1	7,9	6,9	1,7	11,9	9,4	4,0
	4,1	3,0	2,8	6,9	6,4	2,6	7,8	7,8	5,7

The larval numbers of *Cooperia*, *Haemonchus* and *Oesophagostomum* of the group treated with *D. flagrans* recovered from the pasture (per Kg dry matter) reduced by 60.5, 53.2 and 47.5%. In the group treated with *M. thaumasium* the reduction was 33.6, 26.6 and 57.5% respectively (p<0.01). *M. thaumasium* provided a lower percentage reduction of *Cooperia* and *Haemonchus* in the pasture in relation to *D. flagrans*.

In both groups treated, the number of L3 recovered up to 20cm differed from that found 20 and 40cm from the fecal pat. Eighty-four percent of the total L3 recovered from the paddocks were in the distances of up to 20 cm from the fecal pat and only 16% was recovered within 20–40 cm from the fecal pats in the group treated with *D. flagrans*.

The same pattern was found in the group treated with *M. thaumasium*, where 91% of the larvae were up to 20cm from the fecal pat and only 9% from 20 to 40 cm. In the control group the highest percentage of L3 was also up to 20 cm from the fecal pat (73%), but there was a greater amount of L3 recovered from 20 to 40cm (27%) compared with the treated groups.

The percentage reduction of L3 in relation to the group control in the distances of up to 20 and 20–40 cm from the fecal pat was 64.5% and 73%, respectively, for group A, Group B showed percentage reductions of 47.3% and 58%, in the same distances. *D. flagrans* showed larger percentage reduction of L3 in the pasture compared with *M. thaumasium* ($p>0.01$), but no difference was observed between the groups A and B in October, November and December 2010 and January 2011.

The correlation coefficient between mean EPG of each group and infective larvae recovered from the paddocks of group A within the distance from 0 to 20 cm from fecal pats was 0.74 and for the distance between 20 and 40 cm was 0.65. For group B, the correlation coefficient between EPG and infective larvae recovered within 0 to 20 cm from the fecal pats was 0.67 and between 20 and 40 cm was 0.62. For group C, the correlation coefficient recovered within 0 to 20 cm from the fecal pats was -0.68 and within 20 to 40 cm was -0.54. For the treated groups (A and B) there was a strong positive correlation between EPG and infective larvae, although in for group C a negative correlations between EPG and infective larvae was observed.

Figure 3 shows the mean weight gains of animals of the three groups. The weight gain of the animals of the treated groups differed from those of the control group.

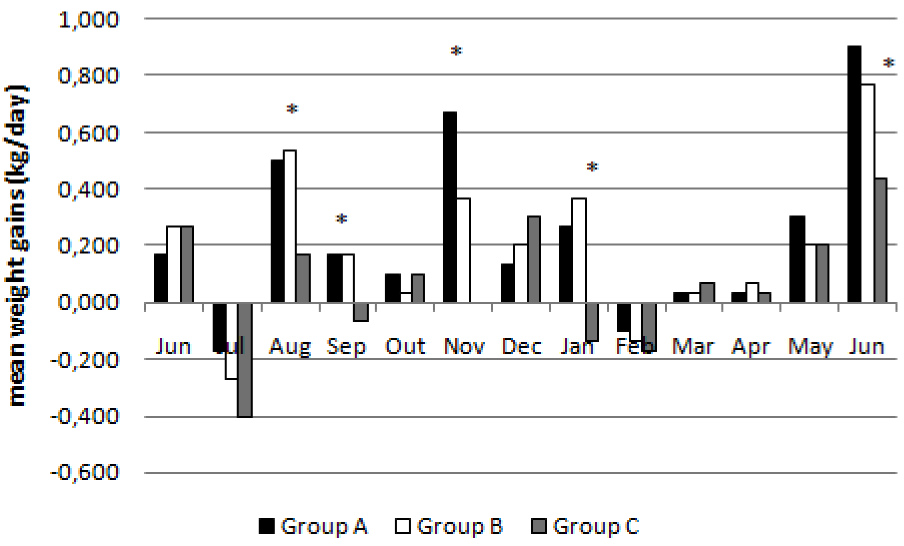
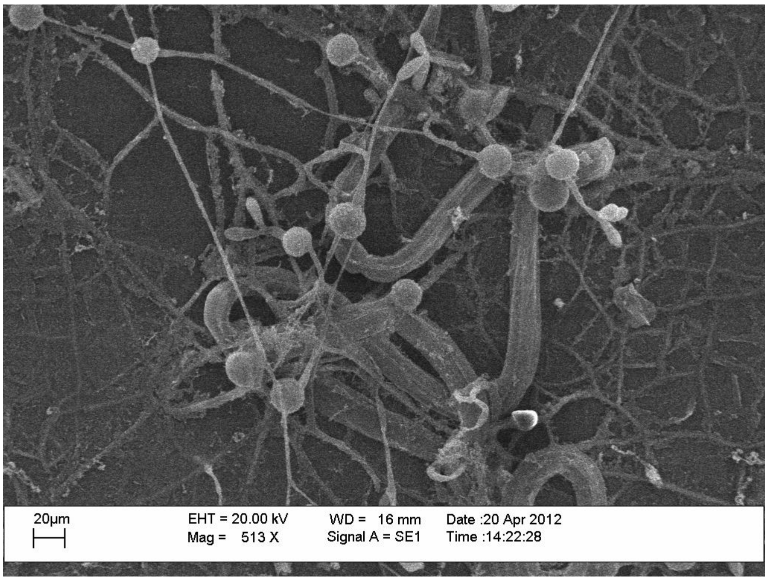


Fig. 3. Mean weight gains (kg/day) of the groups treated with *D. flagrans* and *M. thaumasium* and control from May 2010 to June 2011, Florestal, MG, Brazil. Significant difference (p<0.01) between the treated group and the control denoted by asterisk -Tukey test.

Figure 4 shows scanning electron micrographs of fungi *D. flagrans* (4A) and *M. thaumasium* (4B) recovered from the fecal samples of the treated animals. The analysis of the plates showed the fungal growth and the ability to predate L3 of *D. flagrans* and *M. thaumasium*. Presence of nematophagous fungi was not detected in the feces of the control group during the experiment.



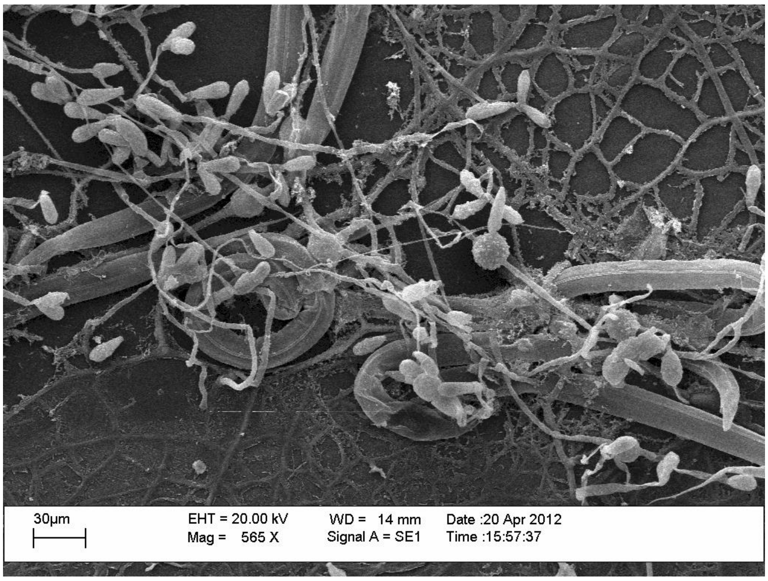


Fig. 4. Scanning electron micrographs of trap formation process and interaction of the fungus *D. flagrans* (Fig. 4A) and *M. thaumasium* (Fig. 4B) with trichostrongyles larvae on dialysis membrane surface.

3.1. Discussion

In the present work, the animals in the group treated with the fungus *D. flagrans* had a reduction in EPG of 56.67%, compared with the animals in the control group. Several works using the fungus *D. flagrans* in ruminants also recorded smaller monthly average values of EPG counts of treated animals in relation to the control group (Dimander et al. 2003; Fontenot et al. 2003; Araújo et al. 2004; Silva et al. 2009). Dias et al. (2007) in a study with the same *D. flagrans* isolate obtained 31% reduction in EPG of treated crossbred Holstein-Zebu cattle.

The group treated with *M. thaumasium* had an EPG reduction of 47.8% in relation to the control group. Studies using the fungus *M. thaumasium* in ruminants recorded monthly average values of EPG counts lower in treated animals. Alves et al. (2004), with crossbred Holstein-Zebu cattle treated with same isolate of *M. thaumasium* obtained 88.8% reduction in EPG after four months of trial.

There are not previous records in the literature of studies involving nematophagous fungi in biological control in cattle during the whole year, going through the dry and rainy seasons in a tropical climate. There are no reports of studies comparing two fungi *in vivo* simultaneously in the same environmental conditions. In this study *D. flagrans* and *M. thaumasium* were proven effective in the reduction of EPG counts in cattle, however, it showed that in these climatic conditions, *D. flagrans* was more effective than *M. thaumasium* in reducing EPG. It was also observed that the isolate of *M.*

thaumasium was less effective in the months with low rainfall (April, May and June 2011). The production of chlamydospores may be related with these results, because it is knew that *D. flagrans* produces more chlamydospores than *M. thaumasium* (Fig. 4).

Araújo et al. (1998) demonstrated that in the southeast region of Brazil, *Cooperia* spp. and *Haemonchus* spp. were the most prevailing gastrointestinal nematodes in bovine followed by *Oesophagostomum* spp. These results are also in accordance with the present work.

Araújo et al. (2007) recorded a larva decrease in the coprocultures of the animals treated with the fungus *M. thaumasium* in the Brazilian semiarid. Larsen et al. (1998) evaluated the potential of the fungus *D. flagrans* in the control of the free living stages of the animal parasites and achieved a reduction of more than 80% in the number of L3 in paddocks. In present work, the effectiveness of the fungi *D. flagrans* and *M. thaumasium* against L3s of *Cooperia*, *Haemonchus* and *Oesophagostomum* was observed in the treated groups. In relation to differential percentage of infective larvae *Cooperia* was the most prevalent genus in all groups. In Brazil, Furlong et al. (1985), Alves et al. (2004) and Araújo et al. (2004) also reported predominance of the genus *Cooperia* in the pasture in comparison with other genera.

Climatic conditions, such as temperature and rainfall favored the development of free-living stages and migration to the herbage (Quinelato et al., 2008). The number of L3 recovered from the paddocks in the distances up to 20 cm and 20 to 40 cm from the fecal pat was similar ($p>0.05$) for the animals in groups A and B in the months with highest rainfall rates. In these months, a larger number of trichostrongyles larvae were recorded in pasture. The lowest rainfall rates were observed in May, June and July 2010 with 48.2, 9.8 and 0 mm^3 in April, May and June 2011 with 7.4, 0.8 and 12 mm^3 respectively, coinciding with a low count of trichostrongyle larvae recovered from the pastures. Once the larvae migrate from feces onto pastures, it is possible that the migration 20 cm beyond the fecal pat is performed by few larvae.

In relation to weight gain, all groups showed continued growth in weight during the rainy season and lost of weight during the dry season, relationed with a marked reduction in production and nutritive value of the pasture. Even though, difference was observed in the weight gain of animals of the treated groups in comparison with the animals of the control group. This reinforces the fact that the administration of pellets with fungi was favored by the treatment of the animals, with reduction of infective larvae in the pasture where the animals of this group were likely to be contaminated by a lower infective larval burden. A higher weight gain in the animals in relation to the control group was also observed by Araújo et al. (2007) when testing the fungus *M. thaumasium* in goats in the Brazilian semiarid. Braga et al. (2009) in a field study with horses using the nematophagous fungus *D. flagrans* observed significant differences in weight gain among the groups treated with the fungus and the control. In the period from March to June the rate of weight increase does not look much different in the 2 groups. There was significant difference ($p>0.01$) for weight gain between the two treated groups only in three months of the study (July, October and November 2010), when the animals treated with *D. flagrans* showed higher mean weight. Regarding the linear coefficients of regression presented in this work, the results showed that there is a positive correlation between the reduction of EPG of the animals and the reduction of L3 in the pastures of the treated group. Dias et al. (2007) reported that there may be dependence between EPG and infective larvae recovered from pasture. In this study, the significant correlations between EPG and L3 in pasture indicate that environmental contamination was an important factor in increasing the parasite load of animals.

3.2. Conclusion

The treatment of beef cattle with alginate pellets containing the nematophagous fungi *D. flagrans* and *M. thaumasium* can be used as an alternative method in the control of bovine gastrointestinal nematodes. The fungus *D. flagrans* was shown more promising than *M. thaumasium* for continuous use during the dry and rainy seasons in tropical regions of low rainfall.

Acknowledgments

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CAPÍTULO 4

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Comparison between biological control with fungi (*Duddingtonia flagrans* and *Monacrosporium thaumasium*) and chemical control (moxidectin) of gastrointestinal nematodiasis in beef cattle of tropical southeastern Brazil

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Abstract

Sodium alginate pellets of two different nematode predatory fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* (biological control) and moxidectin (chemical control) were evaluated in the control of gastrointestinal nematodiasis in bulls. Four groups of ten 6-month-old male Nellore calves were kept in paddocks of *Brachiaria decumbens* for 12 months. Each animal of group A received 1 g/10 kg body weight (b.w.) of pellets of *D. flagrans* (0.2 g fungus/10 kg b.w.) twice per week, for 12 months. In group B, each animal received 1 g/10 kg b. w. of *M. thaumasium* pellets, twice per week, for 12 months. In group C, each animal received 1 ml/50 kg b.w. of the anthelmintic moxidectin (1%) in May, July and September 2010. Animals of the control group (group D) received no treatment. In the analysis of fecal egg count (eggs per g feces; EPG), groups A and B had a 56.67% and 47.8% smaller count, respectively, than that of the control group ($p<0.05$). Moxidectin reduced EPG by 93.8% in the first 6 months of the study (period of application) and by 12.4% in the last 6 months, indicating reinfection by nematodes. Biological control maintained a low EPG in the long term while chemical control resulted in a short-term effect. The percentage reduction of infective third-stage larvae (L_3) in relation to the control group at distances of up to 20 and 20–40 cm from the fecal pat was 64.5% and 73%, respectively, for group A; group B showed percentage reductions of 47.3% and 58% at the same distances. *Duddingtonia flagrans* showed a larger percentage reduction of L_3 in the pasture compared with *M. thaumasium*. Moxidectin showed no reduction of larvae in the pasture. Treatment of bulls with nematophagous fungus and moxidectin integrated in pellets can be used as an alternative treatment of bovine gastrointestinal nematodiasis; however, biological control was more efficient than chemical control over the long term in the environmental conditions of the present study.

KEYWORDS: Cattle Nematoda, control methods of Nematoda, *Duddingtonia flagrans*, *Monacrosporium thaumasium*, moxidectin, helminths, beef cattle

INTRODUCTION

Gastrointestinal parasitism by nematodes causes diseases and can kill ruminants throughout the world, but the greatest economic impact is on the reduction in growth of young animals, resulting in low productivity (Araújo et al., 2007). The indiscriminate use of anthelmintics has provoked the selection of helminth populations that are resistant to the different chemical groups used in cattle treatments (Mota et al., 2003). Besides, the chemical residues of such products have a negative impact on the environment and public health. The growing concern about food and environmental safety has required new strategies in the control of gastrointestinal nematodes in order to reduce the use of synthetic molecules in conventional farming and extensive organic livestock farming.

One of the possible alternatives that have been researched and proposed for use alone or integrated is biological control with predatory fungi of nematodes. Research on the nematode-trapping fungi *Duddingtonia flagrans* (Larsen et al., 1995; Sarkunas et al., 2000; Dias et al., 2007) and *Monacrosporium thaumasium* (Alves et al., 2003; Araújo et al., 2004) with cattle has demonstrated the potential of the organisms as biological control agents against the free-living stages of parasitic nematodes in livestock under experimental and natural conditions. However, most studies of biological control in cattle have been conducted in temperate regions (Larsen et al., 1995; Sarkunas et al., 2000) and with dairy cattle, which are usually placed in the pasture only in the grazing season. There are no previous studies evaluating biological control with *D. flagrans* or *M. thaumasium* in extensive systems of beef cattle and, due to weather conditions, maintained on pasture throughout the year.

Furthermore, there are no studies that have compared the effects of chemical and biological control in reducing nematodes of cattle, allowing the development of an integrated methodology of application of chemical and biological control. Worldwide, there have been several reports on bovine anthelmintic resistance to ivermectin and albendazole, and to the association of ivermectin and albendazole, levamisole (Cleale, 2004; Waghorn et al., 2006). The avermectins (ivermectin and doramectin) and milbemycins (moxidectin) are potent anthelmintic compounds known as endectocide molecules due to their activity against ecto- and endoparasites (Shoop et al., 1995). They have low mammalian toxicity and their formulations are convenient to use. Hence, they are extensively used worldwide in veterinary medicine.

Thus, two isolates of nematophagous fungi native to Brazil and the anthelmintic moxidectin were selected for this study. This is the first work in its field to evaluate the effectiveness of the nematophagous fungi *D. flagrans* and *M. thaumasium* by testing pellet formulations of these fungi in a sodium alginate matrix in the biological control of gastrointestinal nematodiasis in beef cattle reared in an extensive system, comparing their effectiveness with a traditionally used chemical control (moxidectin), under the climatic conditions of tropical southeastern Brazilian.

MATERIALS AND METHODS

Study area

The experiment was carried out on the experimental farm of the Federal University of Viçosa, located in the municipality of Florestal, state of Minas Gerais, southeast region of Brazil (19º53′22″ south latitude and 44º25′57″ west longitude), from May 2010 to June 2011. The paddock's topography is flat to hilly (29% flat, 54% undulating and 17% hilly), with an average altitude of 750 m and native vegetation of the forest–cerrado transition zone. The climate is tropical with a dry season (Köppen–Geiger climate classification: Aw), with an annual average maximum temperature of 28ºC and an average minimum temperature of 13.9ºC.

Fungal cultures

Two isolates of the predatory fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34A) were kept in test tubes containing 2% corn meal–agar (Difco®, USA) at 4ºC in the dark. These isolates came from a Brazilian soil and belong to the mycology collection of the Federal University of Viçosa, Brazil. To induce the formation of fungal mycelium, culture discs of 5 mm in diameter were transferred to 250 ml Erlenmeyer flasks containing 150 ml of potato dextrose (Difco®, USA) liquid medium (pH 6.5) under agitation at 120 revolutions per min, in the dark, at 26ºC, for 10 days. After this period, the mycelia were harvested with a platinum loop, and weighed in an analytic scale for the future production of the pellets, which were made in a sodium alginate matrix according to Walker and Connick (1983) and as modified by Lackey et al. (1993).

Treatment of cattle

A total of 40 male Nellore calves, 6 months old, were previously dewormed with 7.5 ml albendazole/10 kg body weight (b.w.). After 15 days of anthelmintic treatment, the cattle were divided into four comparable groups (A, B, C and D) of 10 animals each. The bulls were weighed and allocated to four 15.0 ha paddocks of *Brachiaria decumbens*, naturally infested with gastrointestinal parasite helminths, due to previous grazing by young and adult animals. Each group was allocated to only one paddock without rotational grazing between the groups during the experimental period. In group A, each animal received 1 g of pellets containing the *D. flagrans* fungus/10 kg b.w., twice per week for a period of 1 year. In group B each animal received 1 g of pellets containing the fungus *M. thaumasium*/10 kg b.w., twice per week for a period of 1 year. In group C, animals were subcutaneously injected with moxidectin (1%), according to the manufacturer's instructions (0.2 mg/kg; Cydectin, Fort Dodge®), in May, July and September 2010. Group D animals, the control group, received no treatment.

Processing of fecal samples

After the introduction of the bulls into the paddocks, samples of feces were collected, once per week, directly from the rectum to determine the number of nematode eggs per g feces (EPG), according to Gordon and Whitlock (1939). Fecal samples were collected from the animals to observe the growth of the fungi, once per week, 2 days after treatment with fungi. The feces were incubated in plates containing 2% water–agar and 100 infective third-stage larvae (L_3) recovered from the coprocultures and put into a drying oven at 25ºC for 10 days to confirm the passage and the predatory ability of the fungi through the gastrointestinal tract of cattle and growth in the feces. At the same time as the EPG examination, coprocultures were carried out for each animal, according to methodology described by Roberts et al. (1952). The identification of the L_3 in the coprocultures was performed according to Keith (1953). EPG and L_3 recovered from coprocultures of animals from both treated and control groups were recorded, and the percentage of larval reduction was determined according to

Mendoza-De-Guives et al. (1999): reduction (%) = mean L_3 recovered from control group − mean L_3 recovered from treated group × 100/mean L_3 recovered from the control group.

Pasture samples

Every 15 days, herbage samples were collected in the paddock of each group, in a zigzag pattern from alternated points, at distances of 20 cm and 20–40 cm from fecal pats, according to Amarante et al. (1996). Then, a 500 g herbage sample was weighed, and parasitic nematode larvae were recovered following the procedure of Lima (1989). The samples were incubated in a drying oven at 100ºC for 3 days to determine the dry matter content. Data were transformed into larvae per kg dry matter.

Treatment of data

Meteorological data were obtained at the specialized station located in the Florestal region. The monthly means of maximum, medium and minimum temperatures, relative humidity and rainfall are reported. EPG, the number of L_3 recovered from the feces and paddocks, and correlation between EPG and recovered L_3 were compared over the experimental period. The weight of the animals was compared during the months of the experiment, starting from June 2010. EPG data were transformed into log (x + 1) prior to analysis. Data were examined by analyses of variance and Tukey's multiple-comparison test with 1% probability.

RESULTS AND DISCUSSION

The monthly average values of EPG counts are shown in Fig. 1. In the first 3 months of the experiment (May, June and July 2010) there was no significant difference ($p>0.05$) between the groups treated with fungi (A and B) and the control group (D). From the month of August, group D (control) presented higher averages for EPG than groups A and B, until the end of the experiment (June 2011). In the last 6 months (January–June 2011) a statistical difference was observed ($p<0.05$) between the groups treated with fungi (A and B) and the moxidectin-treated and control groups (C and D). At the end of the experiment, mean monthly EPG from group A was 56.7% lower than the EPG of the animals of group D (control). In a study with the same *D. flagrans* isolate (AC001), Dias et al. (2007) obtained a 31% reduction in EPG of treated crossbred Holstein–Zebu cattle. In group B, treated with *M. thaumasium*, the reduction in mean EPG compared with the control group was 47.8% (*p*<0.05). Alves et al. (2003), in a study with crossbred Holstein–Zebu cattle treated with the same isolate of *M. thaumasium* obtained an 88.8% reduction in EPG after 4 months of their trial. There was a statistically significant difference (*p*<0.05) between the fungi *D. flagrans* and *M. thaumasium* on August 2010 and February, March, April, May and June 2011. EPG decreased significantly more in *D. flagrans* than in *M. thaumasium* at the end of the experiment.

Group C presented a particular EPG curve during the experiment. In group C, a reduction in EPG count was rapidly observed after the treatment with moxidectin. Unlike groups A and B, which showed a gradual reduction in the elimination of eggs, animals treated with moxidectin showed variations in the curve of EPG over the course of the study. Moxidectin prevents reinfestation by nematodes for a minimum period of 28 days, but does not prevent reinfestation over a longer period of time (Cleale, 2004). Thus, in the first 6 months of the experiment when the animals received doses of moxidectin at monthly intervals (May, July and September 2010), the reduction in mean EPG of group C compared with control was 93.8% (*p*<0.05), but within 6 months of the study EPG reduction was 12.4%, since during these months the animals received no new applications of anthelmintics and were reinfected by nematodes. According to Cleale (2004) the means of fecal EPG counts from cattle treated with injectable moxidectin were reduced by 99.8% at 14 days after treatment, by 99.1% at 28 days after treatment and by 96.7% at 55/56 days after treatment. Soutello et al. (2010), in an experiment with the same drug (Cydectin®, Fort Dodge), obtained a reduction in EPG of 98.3% at 36 hours post-treatment. However, in these studies, the authors did not evaluate the reduction of EPG for a long period after treatment with moxidectin.

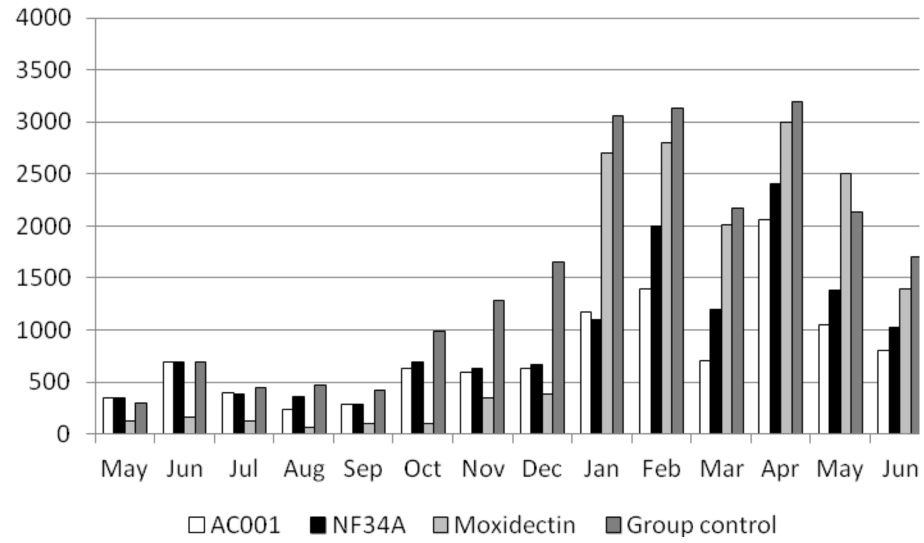

Fig. 1. Monthly average counting of eggs per g feces (EPG) for the groups treated with the nematophagous fungi *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34A) (1 g fungus/10 kg body weight), for the group treated with moxidectin (May, July and September 2010) and for the control group, collected from May 2010 to June 2011 in Florestal, Minas Gerais, Brazil.

The percentage compositions of L_3 of different nematodes obtained after larval culture are presented in Table 1. The prevalence of *Cooperia* sp. was greater than that for the other nematodes in all groups, with percentages of 48.4, 53.1, 69.5 and 44.1% for the groups A, B, C and control, respectively, followed in descending order by *Haemonchus* sp. and *Oesophagostomum* sp. *Haemonchus* sp. were 38.2, 34.7, 17.4 and 43.4% and *Oesophagostomum* sp. were 12.1, 10.9, 11.5 and 10.4%, in the four groups, respectively. Genera found in smaller quantities and sporadically in the four groups were *Bunostomum* sp. (0.8, 0.6, 1.0 and 1.2%) and *Strongyloides* sp. (0.5, 0.7, 0.6 and 0.9%). The results may indicate the presence of moxidectin resistance after treatment (group C). The genus *Cooperia* probably showed resistance to moxidectin, based on larval identification. A difference was observed in the percentages of *Cooperia* sp. in relation to the genera *Haemonchus* and *Oesophagostomum* of the animals in group C (Table 1). During the period when the animals were receiving applications of moxidectin the percentage of *Cooperia* was significantly higher than for other genera in this group.

Table 1. Percentage values corresponding to infective larvae (L_3) recovered from the coprocultures of the groups treated with the nematophagous fungi *Duddingtonia flagrans* (group A) and *Monacrosporium thaumasium* (group B) (1 g fungus/10 kg body weight), treated with moxidectin (May,

July and September 2010) (group C) and of the control group (group D), collected from May 2010 to June 2011 in Florestal, Minas Gerais, Brazil. (Coop. = *Cooperia*, Haem. = *Haemonchus*, Oeso. = *Oesophagostomum*)

	Group A			**Group B**			**Group C**			**Group D**		
	Coop	Haem	Oeso	Coop	Haem	Oeso	Coop	Haem	Oeso	Coop	Haem	Oeso
May	45	55	0	39	61	0	87	4	9	61	39	0
Jun	29	71	0	21	71	4	84	8	8	75	25	0
Jul	62	35	3	72	24	0	75	10	15	41	53	6
Aug	68	27	5	74	18	3	78	14	8	29	63	8
Sep	75	11	14	82	10	2	69	11	20	22	68	10
Out	65	25	10	75	8	17	59	24	13	22	60	18
Nov	76	11	9	77	9	14	55	25	14	17	56	7
Dec	36	8	56	61	29	8	63	27	10	18	52	25
Jan	55	33	12	41	10	49	49	34	15	40	41	19
Feb	44	25	31	61	25	14	77	10	13	35	29	32
Mar	50	34	6	76	4	20	71	8	21	46	49	5
Apr	32	54	14	14	77	9	49	37	8	69	24	7
May	26	64	10	28	67	5	76	19	5	63	28	9
Jun	15	82	0	23	70	7	81	12	2	79	21	0
Average	48.4	38.2	12.1	53.1	34.5	10.9	69.5	17.4	11.5	44.1	43.4	10.4

Another finding indicating resistance of *Cooperia* to moxidectin is presented in the comparison of recovery of this genus relative to other groups in the months from May to October 2010. Although the genus *Cooperia* has been prevalent in the study, in these months there was a distinct emphasis on the percentage of these larvae compared with the percentage found in all the other groups (A, B and D). In group C (moxidectin) *Cooperia* showed no progressive reduction in its recovery, as in the groups treated with fungi (A and B), as the fungi are not selective between nematode larvae and reduced the number of larvae of all genera.

Currently, anthelmintic resistance has become a threat to the prophylaxis of parasitic gastroenteritis in cattle worldwide (Soutello et al., 2007). In Brazil, there are several reports on bovine anthelmintic resistance. A high frequency of ivermectin-resistant *Haemonchus* spp. and *Cooperia* spp. has been detected and in some cases albendazole-resistant nematodes were also found (Soutello et al., 2007; Souza et al., 2008). According to Codin et al. (2009), moxidectin presented 100% efficacy against the genera *Haemonchus* and *Trichostrongylus*, but 65.2% efficacy against *Cooperia* spp. Soutello et al. (2007), in a study with cattle in Brazil, related that moxidectin-resistant *Cooperia* species were detected (*Cooperia punctata* and *C. pectinata*).

Cooperia species are involved in most of the reports concerning anthelmintic resistance in cattle. Reduced moxidectin efficacy against larval stages of *C. punctata* (Williams and DeRosa, 2003) and against adult stages of *C. punctata* (88.4%), *C. spatulata* (93.2%), *C. oncophora* (88.7%) and *C. surnabada* (89.1%) (Ranjan and Delay, 2004) was reported in cattle in the USA. These studies indicate that the capacity of *Cooperia* to survive or develop resistance to the macrocyclic lactone anthelmintics is apparently higher than that of the remaining nematode species. Comparing the fungi *D. flagrans* (group A) and *M. thaumasium* (group B) there was no difference between the nematode genera found. These isolates showed no selectivity for a particular genus.

The larval numbers of *Cooperia*, *Haemonchus* and *Oesophagostomum* recovered from the pasture (per kg dry matter) of the group treated with *D. flagrans* reduced by 60.5, 53.2 and 47.5%. In the group treated with *M. thaumasium* the reduction was 33.6, 26.6 and 57.5%, respectively ($p<0.01$). *Monacrosporium thaumasium* provided a lower percentage reduction in the pasture compared with *D. flagrans*. The group treated with moxidectin showed no reduction in the number of larvae on pasture compared with the control group. Fontenot et al. (2003) reported that *D. flagrans*, in addition to reducing the number of L_3 in the pastures, could also account for the reduction of the L_3 burden in animals that would be introduced into the pasture in the future. Isolates of *Duddingtonia* reduced the level of L_3 in herbage by 74–85% (Grønvold et al., 1993). In all groups, the number of L_3 recovered up to 20 cm differed from that found 20 and 40 cm from the fecal pat. Of the total L_3 recovered from the paddocks, 84% were in the distance of up to 20 cm from the fecal pat and only 16% were recovered within 20–40 cm from the fecal pats in the group treated with *D. flagrans*. The same pattern was found in the group treated with *M. thaumasium*, where 91% of the larvae were up to 20 cm from the fecal pat and only 9% from 20 to 40 cm. Acording to Souza et al. (2008), in the dry season, most of the larvae remain in the stool, or near the base of grass. The opposite occurs during the rainy season when the larvae can migrate 90 cm around the stool. In the moxidectin and control group the highest percentage of L_3 was also up to 20 cm from the fecal pat (70 and 73%), but there was a greater amount of L_3 recovered from 20 to 40 cm (30 and 27%) compared with the treated groups. The percentage reduction of L_3 in relation to the control group in the distances of up to 20 and 20–40 cm from the fecal pat was 64.5% and 73%, respectively, for group A; group B showed percentage reductions of 47.3% and 58%, for the same distances. *Duddingtonia flagrans* showed a larger percentage reduction of L_3 in the pasture compared with *M. thaumasium* ($p>0.01$), but no difference was observed between groups A and B in October, November and December 2010 and January 2011. The group treated with moxidectin showed no statistical difference in the number of recovered larvae on pasture compared with the control group, and thus there was no effect in reducing or preventing reinfestation through pasture. This result was expected since moxidectin acts by reducing the parasitic load of the animals in the short term only and at this point it is appropriate to integrate with a biological control program.

Climatic data are presented in Fig. 2. The monthly average temperature ranged from 15.9°C (July 2010) to 27.2°C (March 2011).

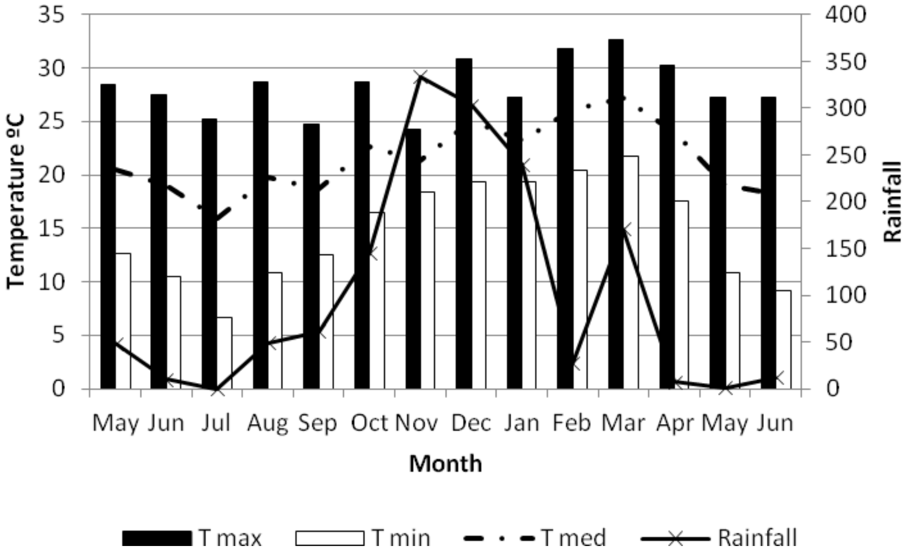

Fig. 2. Monthly average maximum (T max), minimum (T min) and medium (T med) temperatures, and average monthly pluviometric gradient in Florestal, Minas Gerais, May 2010 to June 2011.

The optimum temperatures for the development of most gastrointestinal nematode parasites of cattle (Trichostrongylidae) are between 20 and 30°C (O'Connor, 2006). Average temperatures from 16 to 20ºC are suitable for the development of most parasitic nematode larvae of cattle in pastures. The optimum temperature for the growth and predatory activity of nematophagous fungi is between 20 and 30°C (Su et al., 2007). In the climatic conditions of this study, temperature had no effect in limiting larval development because the average temperatures observed favor the growth and action of the fungal isolate for almost all the experimental period. From May to July 2010 and from April to June 2011 (dry season) rainfall was below 50 mm. The minimum rainfall required for larval development in the environment is 50 mm (Boom and Sheath, 2008). During June 2010 and June 2011, temperature and rainfall were unfavorable for egg and larval development in pastures. Almost no larvae were recovered in the pastures during these months, but even when the rainfall was very low or zero, there was reinfection of the animals, since eggs were recovered in the feces. These results indicate that humidity in the stool allows the development of nematodes in their free-living stages and that low rainfall is sufficient for the development and larval migration to the pastures. Van Dijk et al. (2009) noted that infection levels are highest during the rainy season and related to higher humidity, which favors the development of parasites in their free-living stages and L_3 migration from the stool to adjacent pastures. The low larval occurrence on pasture in periods without precipitation and scores higher in the rainy season observed in this study are consistent with these observations. In months where temperature and humidity are not favorable for the development of fungus and nematode larvae on pasture, it would not be necessary to use the fungus. This study raises the possibility that in the case of consortium application of chemical and biological control, perhaps the use of biological control could be omitted in the months of the initial chemical applications, when the anthelmintic in question is able to reduce infective load by about 90%. However, taking into account the issue of the resistance of some genera to anthelmintics, the ideal would be to not omit the application of fungus. This study highlights the need for future assessments of biological control syndicated to chemical control, at different dosages and climatic conditions.

In relation to weight gain, all groups showed continued growth in weight during the rainy season and lost weight during the dry season, associated with a marked reduction in growth and nutritive value of the pasture (Fig. 3). Even so, a difference was observed in the weight gain of animals of the treated groups in comparison with the animals of the control group. This reinforces the fact that the administration of pellets with fungi favored the health of the animals, with reductions of L_3 in the pasture where the animals of this group were likely to be contaminated by a lower L_3 burden. A higher weight gain in the animals in relation to the control group was also observed by Araújo et al. (2007) when testing the fungus *M. thaumasium* in goats in a semi-arid region of Brazil. Braga et al. (2009) in a field study with horses using the nematophagous fungus *D. flagrans* observed significant differences in weight gain between the groups treated with the fungus and the control group. In the present study, in the period from March to June the rate of weight increase did not appear to be much–_different in the two fungus-treated groups. There was a significant difference (p>0.01)–_for weight gain between the two treated groups only in 3 months of the study (July,–_October and November 2010), when the animals treated with *D. flagrans* showed a higher mean weight. The group treated with moxidectin showed greater weight gain than the groups treated with the fungus in the months of May, July and September 2010, which were the months in which calves received applications of the anthelmintic. According to the data of Cleale (2004), the rate of weight gain by cattle treated with long-acting injectable moxidectin was 0.59 kg/day or 23% (0.11 kg/day) more than controls.

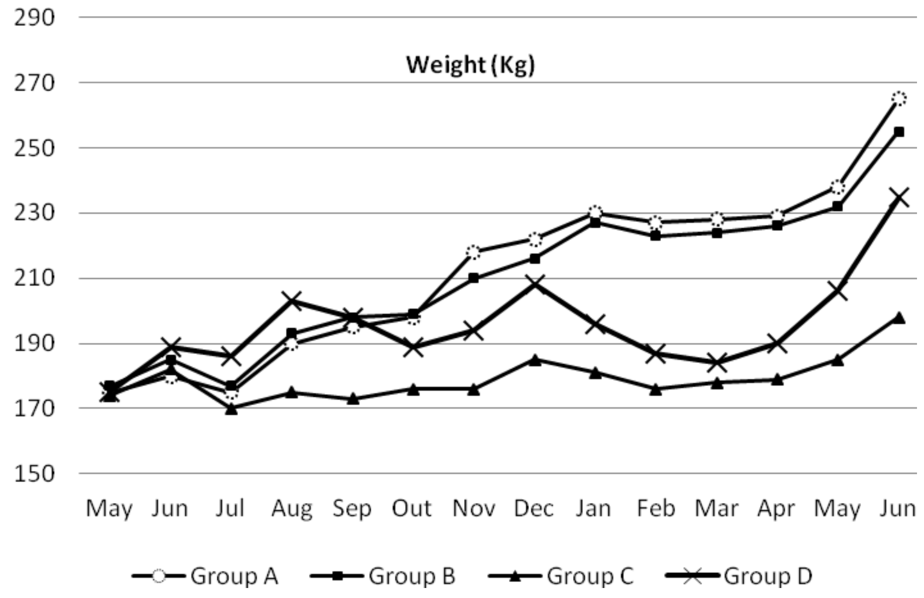

Fig. 3. Mean weight gain (kg/day) of the groups treated with *Duddingtonia flagrans* (group A), *Monacrosporium thaumasium* (group B), moxidectin (group C) and of the control group (group D) from May 2010 to June 2011, Florestal, Minas Gerais, Brazil.

This study raises the possibility that in the case of consortium application of chemical and biological control, perhaps the use of biological control could be omitted in the months of the initial chemical applications, when the anthelmintic in question is able to reduce the infective load by about 90%. On the other hand, taking into account the issue of resistance among genera to anthelmintics, the ideal would be to not omit the application of fungus. This study brings the need for future assessments of biological control syndicated to chemical control, at different dosages and climatic conditions. According to Waller and Larsen (1993), the application of fungi in nematode biocontrol helps the chemical control and should be administered not only when there is a prediction of greater pasture infestation by helminth eggs and larvae, but also when there would be better conditions for the fungi growth in the environment, this way preventing the clinical parasitism and productivity losses, already supplying a sufficient number of larvae to allow these animals to develop a naturally acquired immunity. The results were analysed based on the overall experimental period and we conclude that this dose and periodicity of application of fungus pellets makes the use of other anthelmintic treatments unnecessary.

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CAPÍTULO 5

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Interaction between the nematophagous fungus *Duddingtonia flagrans* and infective larvae of *Haemonchus placei* (Nematoda: Trichostrongyloidea)

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Abstract

The interaction between *Duddingtonia flagrans* and infective larvae of *Haemonchus placei* was studied *in vitro* under optical and scanning electron microscopy. The experimental assay was carried out on plates with 2% water-agar (2% WA), each plate contained the isolate and 1.000 L3. Scanning electron micrographs were taken 12, 24, 36 and 48 h after larval predation. Trap formation by the fungus and first larvae preyed were observed 12 hours after inoculation. Chlamydospores formation was observed in all times analyzed. The amount of conidia increased proportionally with time. Details of predation structures and fungus larvae interaction are described. Cuticle penetration by fungus hyphae occurred only 48 h after predation.

Keywords: *D. flagrans*, *Haemonchus placei*, Scanning Electron Microscopy

Introduction

Many nematode-trapping fungi capture nematodes using an adhesive present on specific capture organs. This fungus produces adhesive three-dimensional net-type traps that capture and destroy the pre-parasitic stages of several nematode species (Barron, 1977; Larsen et al., 1995; Campos et al., 2008). *Duddingtonia flagrans* is considered the most promising species in for biological control against gastrointestinal nematode parasites of livestock (Larsen et al., 1995; Fontenot et al., 2003; Assis et al. 2012). It has been used successfully in several laboratories studies (Braga et al.; 2008) and has proven action *in vivo* against ruminant gastrointestinal nematodes (Larsen et al., 1995; Assis et al., 2012), horses (Baudena et al., 2000; Braga et al., 2009) and goats (Vilela et al., 2012).

Parasitic nematodes of the genus *Haemonchus* infect a range of ruminant hosts and are of major veterinary and economic importance. *H. placei* is a highly pathogenic nematode that affects cattle (Brasil et al., 2012). Along with the genus *Cooperia* spp. *H. placei* is one of the most frequent parasites in Brazil. Produces high rates of morbidity and consequently important economic losses. It hematophagous, producing lesions abomasal mucosa that can cause loss of blood and plasma into the intestinal lumen. Massive infections can lead young animals died of acute anemia and hypoproteinemia (Guimarães et al., 2000; Brasil et al., 2012).

In spite of this research, there is little information about the ultra structural aspects of the interaction between this fungus specie and infective larvae of gastrointestinal nematode parasites in ruminants. The characterization of processes involving fungus-nematode interaction is essential and may influence the selection of isolates for use in biological control programs (Mendoza-de-Gives et al., 1999; Campos et al., 2008).

The objective of this study was to investigate interaction processes between the nematode predator fungus *Duddingtonia flagrans*, isolate AC001, and infective larvae of *Haemonchus placei*, using scanning electron microscopy.

2. Materials and methods

2.1. Fungus

AC001 isolate of nematophagous fungus *D. flagrans* was used. The isolate was stored in test tubes containing 2% corn-meal-agar (2% CMA), in the dark, at 4°C for 10 days. This isolate was obtained from Brazilian soil, in Viçosa city, region of Minas Gerais state. It was collected using the soil-sprinkling method of Duddington (1955), modified by Santos et al. (1991).

2.2. Infective larvae of *Haemonchus placei*

Faeces were collected directly from the rectum of cattle naturally infected with trichostrongyles. These animals were from the Federal University of Viçosa, in Florestal city, Minas Gerais state, Brazil. After that, a count of eggs per gram of feces (EPG) was made according to Gordon and Whitlock (1939), in order to find positive animals. Then coprocultures using fragmented industrial vermiculite (autoclaved) and water were performed. Faecal samples of 20 g each were mixed with 8 g vermiculite and 20 ml distilled water and incubated for 8 days at 25°C in darkness. Larvae were recovered from the culture in tubes used for haemolysis, using a Baermann funnel and water, initially at 42°C, for 12 h. The larval solution was filtered to eliminate debris and to recover the active larvae, using the methodology described by Barçante et al. (2003). The L3s were identified and quantified according to the criteria described by Bevilaqua et al. (1993) using an optical microscope with a 10x objective. To estimate the total larval number, five 20 ml aliquots of larval solution were counted and total larvae in the suspension were estimated.

2.3. Scanning Electron Microscopy

The Nordbring-Hertz (1983) technique, with modifications, was used to prepare the material for scanning electron microscopy. Dialysis membrane discs (Sigma, St. Louis, Missouri, USA), 6 cm in diameter, were cut and placed in Erlenmeyer flasks containing distilled water. The material was autoclaved at 121°C for 15 min. Discs were removed from flasks using tweezers, and placed on the surface of 2% AW in 6 cm diameter Petri dishes, so that membrane edges covered all the agar surface and were raised and adhered to plate edges to prevent larvae passing to the underside of the membrane. Fragments of 1.7% CMA (Corn Meal Agar, Difco, USA) containing mycelia and spores of *D. flagrans* (isolate AC001) were removed from the culture tubes and replicated in three 9 cm diameter Petri dishes containing 20 ml CMA medium. Petri dishes were incubated in a biochemical oxygen demand (BOD) incubator (FANEM, Brazil) at 25°C in darkness. After 7 days, approximately 4 mm diameter fragments were replicated from the edges of contamination-free colonies on to the surface of dialysis membranes (Nordbring-Hertz, 1983). Petri dishes were then incubated in a BOD at 25°C in darkness.

Seven days after incubation, Petri dishes were removed from the incubator and suspension droplets containing approximately 1000 infective larvae of *H. placei* were placed over *D. flagrans* cultures grown on the surface of dialysis membranes. An equal number of larvae were placed into five Petri dishes containing AW without fungus and used as a control of larval viability. In the first 12 h after nematode inoculation on Petri dishes, these cultures were removed from the incubator (25°C in darkness) and observed hourly under optical microscopy (light field) with 100 £ magnification. Predated nematodes in a determined area of Petri dishes were marked on the underneath of the plate with a permanent marker, and each plate was numbered in order to time the fungus–larvae interaction and facilitate the finding of areas with predated nematodes. After predation, observations were made under optical microscopy with 100 £ magnification at 6-hourly intervals. Pieces of dialysis membranes with samples of larvae at stage L3 exposed to capture for 12, 24, 36 and 48 hours were cut with a scalpel and collected with fine pointed tweezers and fixed in 2.5% glutaraldehyde in 0.05M phosphate buffer, pH 7.4 for 24 h, washed six times in the same buffer and dehydrated by passing the material through an ethanol series (30, 50, 60, 70, 95 and 100%). The material was dried to critical point using carbon dioxide, coated with gold and observed using a LEO scanning electron microscope at 10-15 kV, at the Centre for Electron Microscopy and Microanalysis of the Federal University of Viçosa.

3. Results and Discussion

The specific structures (conidia and traps) of predator fungi attached to nematodes were noticed in all Petri dishes (Fig. 1a). In addition, chlamydospores of fungus *D. flagrans* also were observed on Petri dishes (Fig. 1b). Hyphal ramifications were observed 12 h (first observation) after inoculating nematodes on to Petri dishes, showing differentiation of predation structures (Fig. 1c and d). This finding was similar of Campos et al. (2008) that observed hyphal ramifications of *D. flagrans* (isolate CG722) in study with *H. contortus* 9 hours after inoculated larvae. First, these structures were concentrated in areas of greater nematode concentration and later spread all over the membrane surface. The morphology of predation structures changed gradually and, at 24 h, most presented three-dimensional net formation (Fig. 1c and d).

For predator fungi *D. flagrans* (AC001) was noticed by light microscope the interaction of predator fungi in the first 6 h of the test with *H. placei*. The time necessary for trap formation after adding nematodes to plates recorded in the present experiment was similar that reported by Silva et al. (2011) who observed trap presence 6 h after adding 1000 *H. contortus* of L3 on the surface of Petri dishes containing the same isolate AC001. In predator fungi, the transition from saprophytic growth to parasitic stage, when traps are formed, is influenced by biotic and abiotic factors. In the presence of nematodes, mycelia and spores are induced to form structures that will eventually capture nematodes (Nordbring-Hertz, 1988). Larval predation by nematophagous fungi involves several steps (Campos et al. 2008). Adhesion between nematodes and fungi starts as a contact between the surfaces of larval cuticle and trap. In this study, 12 h after adding larvae to plates the first predated larvae were observed numerous points of adhesion of larval cuticle (Fig. 2a). Larvae were found adhering to the surface of the predation structures in all times analyzed (Fig. 2). Points of larval capture varied, and there were no sites in the cuticle specific for hyphal adhesion, besides, in some cases, larvae were captured by several predation structures at different sites of the cuticle surface. In 24 h was observed the presence of conides (Fig. 2b). In 36 h was observed a large formation of constriction rings, tridimensional traps and chlamydospores (Fig. 2c). After capture, hyphae penetrate the cuticle, colonize the interior of the body and emerge again on the surface of the nematode cuticle, producing spores and mycelia (Barron, 1977). In the present study, observations of fungus-larvae interaction for 48 h showed invasion of larval cuticle by the fungus (fig. 2d).

Ultra structural studies by transmission electron microscopy or other microscopical techniques may help to clarify the penetration phase of *H. placei* larvae by *D. flagrans*. Previous studies on fungus–nematode interaction have demonstrated that fungi showing earlier production with greater number of traps and greater aggression against several nematode species could be selected for biological control programs. Molecular and biochemical studies of factors involved in the interaction between *D. flagrans* and infective larvae of gastrointestinal nematode parasites in ruminants could bring important insights, allowing the identification of substances involved in the interaction, with implications for selection of isolates to be used in nematode biological control programs.

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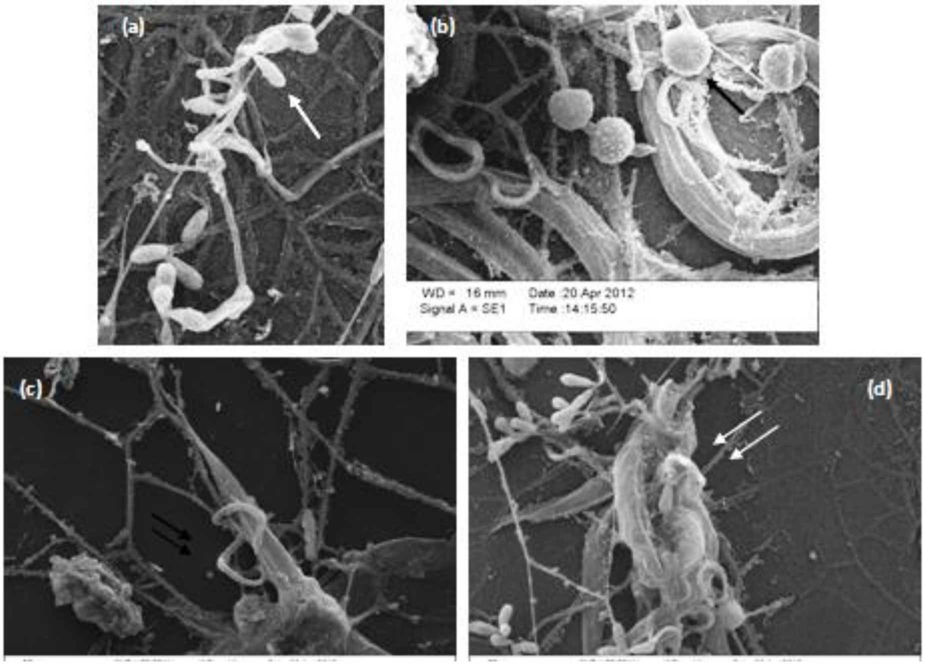

Fig. 1. Scanning electron micrographs of trap formation process and interaction of the fungus *D. flagrans* (AC001) with infective larvae of *Haemonchus placei* on dialysis membrane surface. (a) Conidia (white arrow) 10 µm; (b) Production of chlamydospores (black arrow) 30 µm; (c) three-dimensional adhesive nets (black double arrow) 30 µm; (d) traps (white double arrow) 20 µm.

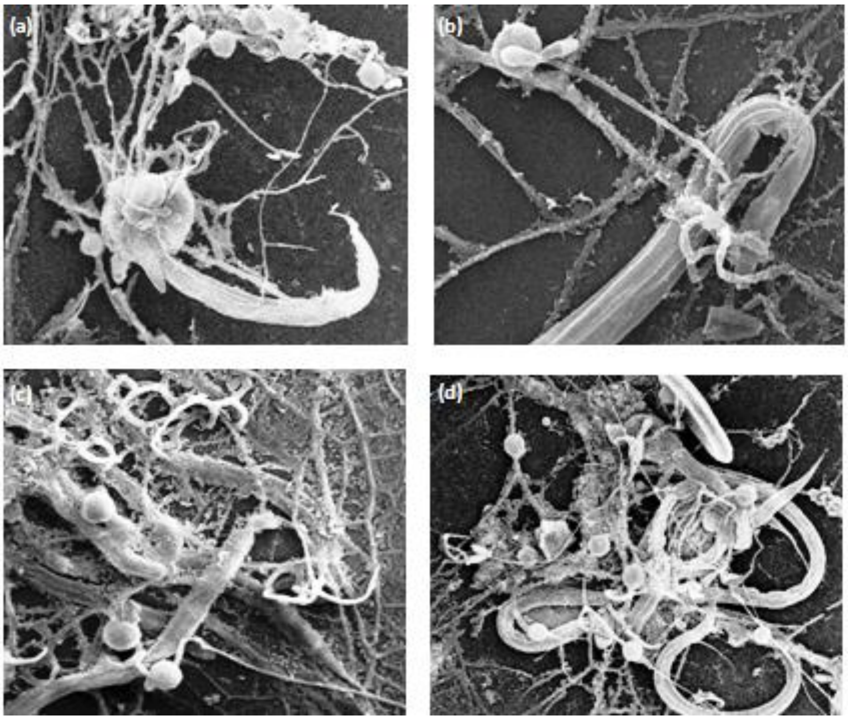

Fig 2. Infective larvae (L3) of preyed *H. placei* by predator fungi *D. flagrans* (AC001). (a) Traps adhering to different surface regions of a stage L3 larvae 12 h after predation; (b) Adherence between traps and nematode cuticle 24 h after predation. (c) Presence of constriction rings, tridimensional traps and chlamydospores within 36 h of nematode-fungus interaction; (d) L3 larva cuticle close to the adherence zone of the trap, 48 h after predation.

CAPÍTULO 6

Interaction between *Monacrosporium thaumasium* and infective larvae of *Haemonchus placei* (Nematoda: Trichostrongyloidea)

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Abstract

The interaction between *Monacrosporium thaumasium* and infective larvae of *Haemonchus placei* was studied *in vitro* under optical and scanning electron microscopy. The experimental assay was carried out on plates with 2% water-agar (2% WA), each plate contained the isolate and 1.000 L3. Scanning electron micrographs were taken 12, 24, 36 and 48 h after larval predation. The first larva predated by the fungus was observed already in the first time (12 h). In all times analyzed at observed the evolution of larval-fungus interaction with details of predation structures, adhesive networks and one conidia at end of conidiophore. The genus *Monacrosporium* does not produce a high quantity of chlamydospores but was possible to identify them in 36 and 48 times.

Keywords: scanning electron microscopy, nematode, predatory fungi

Introduction

Parasitic nematodes of the genus *Haemonchus* infect a range of ruminant hosts and are of major veterinary and economic importance. *H. placei* is a highly pathogenic nematode that affects cattle (Brasil et al., 2012). Along with the genus *Cooperia* spp. *H. placei* is one of the most frequent parasites in Brazil. *H. placei* produces high rates of morbidity and consequently important economic losses. It hematophagous, producing lesions abomasal mucosa that can cause loss of blood and plasma into the intestinal lumen and massive infections can lead young animals died of acute anemia and hypoproteinemia (Guimarães, 2000). One alternative to achieve control of parasites of ruminants, inclusive the *H. placei*, is biological control using nematophagous fungi (Araújo et al., 2004; 2007). The nematophagous fungi can be divided into predators, endoparasites, opportunistic (parasitic eggs, cysts and sedentary females) and those that produce metabolites toxic to nematodes (Campos et al., 2008). Predatory fungi form traps of different types: non-modified adhesive hyphae; hyphal branching anastomosed forming networks adhesive two-dimensional and three-dimensional, adhesive branches formed by one or more cells, lymph adhesives; constrictor rings and rings not constricting (Ribeiro et al., 1999). The biological control studies with nematophagous fungi have been restricted to the *Duddingtonia flagrans* fungus due to the production of chlamydospores, structures having a greater resistance to the conditions imposed by the gastrointestinal tract of animals, however, several works performed in Brazil show that the species belonging to the genera *Arthrobotrys* and *Monacrosporium* are often found in the country (Naves and Campos, 1991; Ribeiro et al., 1999). In studies conducted using fungi of the genus *Monacrosporium* Araújo et al. (2004; 2007) and Assis et al. (2013) observed the passage through the gastrointestinal tract of ruminants with capacity to preyed gastrointestinal nematode parasites on pasture. It is of great importance to analyze the efficiency of native fungal isolates the local soil, avoiding the entry of foreign organisms to the environment and enhancing the reduction of chemical applications for the control of parasites in animals. In spite of this research, there is little information about the ultra structural aspects of the interaction between this fungus specie and infective larvae of gastrointestinal nematode parasites in ruminants. The characterization of processes involving fungus-nematode interaction is essential and may influence the selection of isolates for use in biological control programs (Mendoza-de-Gives et al., 1999; Campos et al., 2008). The objective of this study was to investigate interaction processes between the nematode predator fungus *Monacrosporium thaumasium*, isolate NF34A, and infective larvae of *Haemonchus placei*, using scanning electron microscopy.

2. Materials and methods

2.1. Fungus

NF34A isolate of nematophagous fungus *M. thaumasium* was used. The isolate was stored in test tubes containing 2% corn-meal-agar (2% CMA), in the dark, at 4°C for 10 days. This isolate was obtained from Brazilian soil, in Viçosa city, region of Minas

Gerais state. It was collected using the soil-sprinkling method of Duddington (1955), modified by Santos et al. (1991).

2.2. Infective larvae of *Haemonchus placei*

Faeces were collected directly from the rectum of cattle naturally infected with trichostrongyles. These animals were from the Federal University of Viçosa, in Florestal city, Minas Gerais state, Brazil. After that, a count of eggs per gram of feces (EPG) was made according to Gordon and Whitlock (1939), in order to find positive animals. Then coprocultures using fragmented industrial vermiculite (autoclaved) and water were performed. Faecal samples of 20 g each were mixed with 8 g vermiculite and 20 ml distilled water and incubated for 8 days at 25°C in darkness. Larvae were recovered from the culture in tubes used for haemolysis, using a Baermann funnel and water, initially at 42°C, for 12 h. The larval solution was filtered to eliminate debris and to recover the active larvae, using the methodology described by Barçante et al. (2003). The L3s were identified and quantified according to the criteria described by Keith (1953) using an optical microscope with a 10x objective. To estimate the total larval number, five 20 ml aliquots of larval solution were counted and total larvae in the suspension were estimated.

2.3. Scanning Electron Microscopy

The Nordbring-Hertz (1983) technique, with modifications, was used to prepare the material for scanning electron microscopy. Dialysis membrane discs (Sigma, St. Louis, Missouri, USA), 6 cm in diameter, were cut and placed in Erlenmeyer flasks containing distilled water. The material was autoclaved at 121°C for 15 min. Discs were removed from flasks using tweezers, and placed on the surface of 2% AW in 6 cm diameter Petri dishes, so that membrane edges covered all the agar surface and were raised and adhered to plate edges to prevent larvae passing to the underside of the membrane. Fragments of 1.7% CMA (Corn Meal Agar, Difco, USA) containing mycelia and spores of *M. thaumasium* (isolate NF34A) were removed from the culture tubes and replicated in three 9 cm diameter Petri dishes containing 20 ml CMA medium. Petri dishes were incubated in a biochemical oxygen demand (BOD) incubator (FANEM, Brazil) at 25°C in darkness. After 7 days, approximately 4 mm diameter fragments were replicated from the edges of contamination-free colonies on to the surface of dialysis membranes (Nordbring-Hertz, 1983). Petri dishes were then incubated in a BOD at 25°C in darkness.

Seven days after incubation, Petri dishes were removed from the incubator and suspension droplets containing approximately 1000 infective larvae of *H. placei* were placed over *M. thaumasium* cultures grown on the surface of dialysis membranes. An equal number of larvae were placed into five Petri dishes containing AW without fungus and used as a control of larval viability. In the first 12 h after nematode inoculation on Petri dishes, these cultures were removed from the incubator (25°C in darkness) and observed hourly under optical microscopy (light field) with 100 £ magnification. Predated nematodes in a determined area of Petri dishes were marked on the underneath of the plate with a permanent marker, and each plate was numbered in order to time the fungus–larvae interaction and facilitate the finding of areas with predated nematodes. After predation, observations were made under optical microscopy with 100 £ magnification at 6-hourly intervals. Pieces of dialysis membranes with samples of larvae at stage L3 exposed to capture for 12, 24, 36 and 48 hours were cut with a scalpel and collected with fine pointed tweezers and fixed in 2.5% glutaraldehyde in 0.05M phosphate buffer, pH 7.4 for 24 h, washed six times in the same buffer and dehydrated by passing the material through an ethanol series (30, 50, 60, 70, 95 and 100%). The material was dried to critical point using carbon dioxide, coated with gold and observed using a LEO scanning electron microscope at 10-15 kV, at the Centre for Electron Microscopy and Microanalysis of the Federal University of Viçosa.

3. Results and Discussion

Among various species of nematodes predatory fungi, the genus *Monacrosporium* have been subject of several studies (Araújo et al., 2004). The *Monacrosporium* fungi were sorted by Cooke and Dickson as belonging to the subdivision Deuteromycotina. The specie *M. thaumasium* prey nematodes by adhesive networks and the specific structures (conidia and traps) of predator fungi attached to nematodes were noticed in all Petri dishes analyzed (Fig. 1a). The genus *Monacrosporium* does not produce a high quantity of chlamydospores. *M. thaumasium* produces chlamydospores with 27-49 mm length and 15-23mm wide (Liu & Zhang, 1994). According Saxena & Mittal (1995) the presence of chlamydospores is observed in plate cultures with solid culture limiting. Chlamydospores of fungus *M. thaumasium* were identified in this study (Fig. 1b). *Monacrosporium* species are characterized by producing one conidia at end of the conidiophore. The conidia are hyaline and fusiform measuring between 27 to 49 mm in length, 15-23 mm height with two to four transverse septa and the intermediate cell is bigger than the edge cells (Fig. 1a). The conidiophore is branched near the edge; each branch ends in conidia (Mota et al., 2003). Hyphal ramifications with differentiation of predation structures were observed 12 h (first observation in SEM) after inoculating nematodes on to Petri dishes (Fig. 1c and d). This finding was similar of Campos et al. (2008) that observed hyphal ramifications with predation structures of *D. flagrans* (isolate CG722) in study with *H. contortus* 9 hours after inoculated larvae. First, these structures were concentrated in areas of greater nematode concentration and later spread all over the membrane surface.

In this study, with fungi *M. thaumasium* (NF34A) was noticed by light microscope the interaction of predator fungi in the first 6 h of the test with *H. placei*. The time necessary for trap formation after adding nematodes to plates recorded in the present experiment was similar that reported by Silva et al. (2011) who observed trap presence 6 h after adding 1000 *H. contortus* of L3 on the surface of Petri dishes containing the *D. flagrans* isolate AC001. Several factors may be involved in the processes of formation of different types of trap. These may be produced in response to stimulus caused by the movement of nematodes (Campos et al., 2008), substances excreted by the nematodes (Nordbring-Hertz, 1988), lack of nutrients and water (Nordbring-Hertz, 1983). The process of capture of nematodes by fungi starts with the attraction of nematodes in the traps. In the opinion of Nodbring-Hertz (1983), the presence of the nematode is essential for that formed structures capture. Larvae were found adhering to the surface of the structures in predation in all times analyzed (Fig. 2). Referring to Fig. 2a, we see the start of the formation of a network of adhesive *M. thaumasium*. The capture of infective larvae of *H. placei* in the network adhesive *M. thaumasium* is observed in Fig. 2b. In Fig. 2c, were illustrated points of larval capture varied, and there were no sites in the cuticle specific for hyphal adhesion, besides, in some cases, larvae were captured by several predation structures at different sites of the cuticle surface. We were observed in 24 large presence of conidia and high differentiation traps (Fig. 2b). According Saxena & Mittal (1995) the differentiation process of traps occurs within 24 hours after the fungus and nematode interactions. The higher motility of nematodes increases the stimulus the fungus for the production of traps and the traps formation can be related to the increase of the amount of conidia (Mendoza-de-Gives, 1999). In 36 h were observed large formation of constriction rings, traps and chlamydospores tridimensional (Fig. 2c). After capture, hyphae penetrate the cuticle, colonize the interior of the body and emerge again on the surface of the nematode cuticle, producing spores and mycelia (Barron, 1977). In the present study, observations of fungus-larvae interaction for 48 h showed invasion of larval cuticle by the fungus (fig. 2d). After the seizure of the nematode, this cuticle is damaged and penetrated by a bulb, and then destroyed the nematode and its contents absorbed by the fungus (Campos et al., 2008). Studies suggest that some antigenic differences present in the cuticle of nematodes or in different isolates of the same species could result in different efficacies of predation. The adhesion mechanisms and destruction have not been well characterized in interactions of fungi and nematode parasites of domestic animals, and this characterization of great importance and may substantially affect the ability of a fungus to capture nematodes, with implications in the selection of the isolate to be used in biological control programs (Mendoza-de-Gives et al., 1999). Ultra structural studies by transmission electron microscopy or other microscopical techniques may help to clarify the penetration phase of *H. placei* larvae by *M. thaumasium*. Previous studies on fungus–nematode interaction have demonstrated that fungi showing earlier production with greater number of traps and greater aggression against several nematode species could be selected for biological control programs. Molecular and biochemical studies of factors involved in the interaction between *M. thaumasium* and infective larvae of gastrointestinal nematode parasites in ruminants could bring important insights, allowing the identification of substances involved in the interaction, with implications for selection of isolates to be used in nematode biological control programs.

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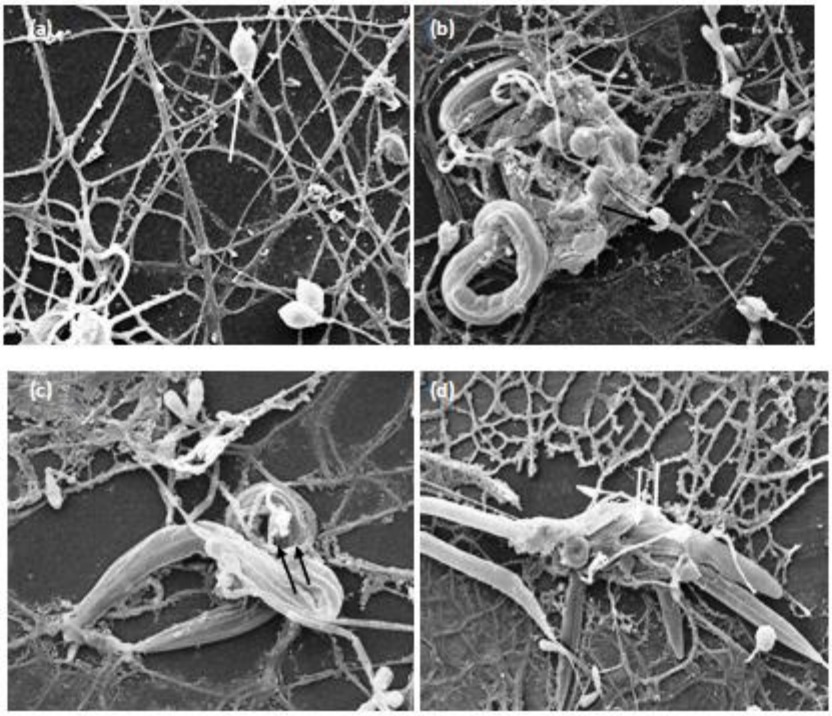

Fig. 1. Scanning electron micrographs of trap formation process and interaction of the fungus *M. thaumasium* (NF34A) with infective larvae of *Haemonchus placei* on dialysis membrane surface. (a) Conidia (white arrow) 30 µm; (b) Production of chlamydospores (black arrow) 30 µm; (c) three-dimensional adhesive nets (black double arrow) 30 µm; (d) traps (white double arrow) 20 µm.

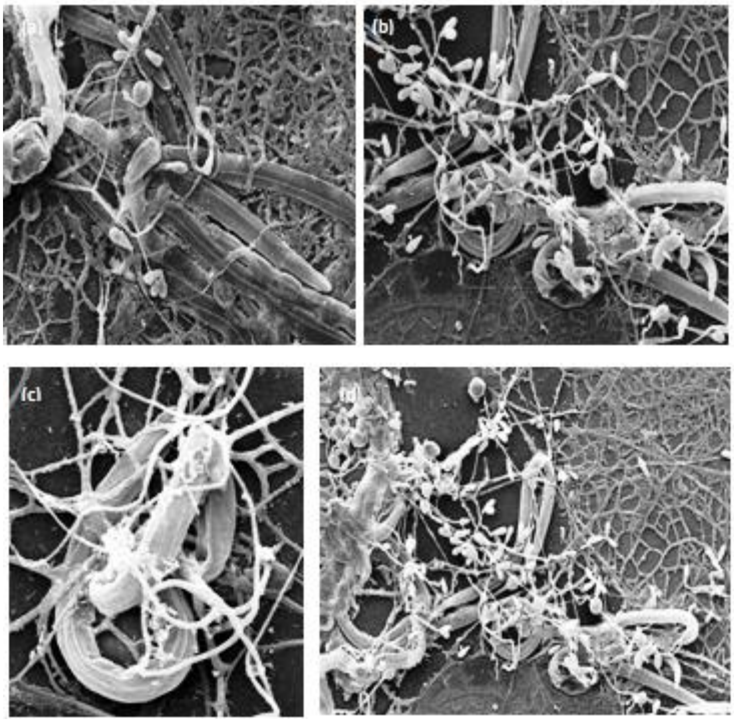

Fig 2. Infective larvae of preyed *H. placei* by predator fungi *M. thaumasium* (NF34A). (a) Presence of formation of constriction rings, tridimensional traps and chlamydospores within 12h of nematode-fungus interaction, 30 µm; (b) Adherence between traps and nematode cuticle 24h after predation, 100 µm; (c) Traps adhering to different surface regions of a stage L3 larva 36h after predation, 20 µm; (d) L3 cuticle close to the adherence zone of the trap, 48h after predation, 200 µm.

CONCLUSIONS

1. The treatment of beef cattle with alginate pellets containing the nematophagous fungi *D. flagrans* and *M. thaumasium* can be used as an alternative method in the control of bovine gastrointestinal nematodes. The fungus *D. flagrans* was shown more promising than *M. thaumasium* for continuous use during the dry and rainy seasons in tropical regions of low rainfall.

2. This study raises the possibility that in the case of consortium application of chemical and biological control, perhaps the use of biological control could be omitted in the months of the initial chemical applications, when the anthelmintic in question is able to reduce the infective load by about 90%.

3. The animals treated with nematophagous fungi had a higher weight gain when compared to cattle treated with moxidectin and those who received no treatment.

4. The interaction between nematophagous fungi *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34A) and infective larvae of *Haemonchus placei* was proven in Scanning Electron Microscopy.