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FUNGOS NEMATÓFAGOS PREDADORES NO CONTROLE DE ANCILOSTOMATÍDEOS DE CÃES

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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"Eu espero que eu sempre possua firmeza e virtude suficientes para manter o que eu considero o mais invejável de todos os títulos, o caráter" (George Washington)

A Deus,
À minha querida mãe,
Dedico.

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BIOGRAFIA

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RESUMO

MACIEL, Alessandro Spalenza, D. Sc., Universidade Federal de Viçosa, Julho de 2009. **Fungos nematófagos predadores no controle de ancilostomatídeos de cães.** Orientador: Jackson Victor de Araújo. Co-orientadores: Artur Kanadani Campos, Laércio dos Anjos Benjamin e Leandro Grassi de Freitas.

Os nematóides do gênero *Ancylostoma* são endoparasitas cosmopolitas de cães que causam diversas patologias nesses animais e também são geohelmintos zoonóticos que podem infectar o ser humano via solo. O controle do estágio adulto destes nematóides é baseado na utilização de anti-helmínticos, no entanto, o uso de agentes biocontroladores pode ser uma medida complementar reduzindo a população dos estágios pré-parasitários em desenvolvimento no solo. Isto se justifica pelo fato de que cães vadios parasitados são os principais responsáveis pela infestação ambiental com ovos de ancilostomídeos veiculados nas fezes. Dentre os organismos biocontroladores, sabe-se que os fungos nematófagos predadores têm tido eficácia contra os nematóides de animais domésticos. Neste contexto, o objetivo deste trabalho foi testar diversos isolados de fungos predadores, selecionando o mais infectivo para ser utilizado no controle da forma larval infectante (L_3) de *Ancylostoma* spp. no solo sob condições semi-naturais. O fungo *Duddingtonia flagrans* (Isolado CG768) foi considerado o mais infectivo devido à redução do número de L_3 recuperadas e pela característica de produzir numerosos esporos de resistência capazes de sobreviver no solo, prolongando a vida do fungo na ausência da presa. A infectividade deste isolado pôde ser observada em micrografias eletrônicas de varredura que mostraram a destruição completa da L_3 de *Ancylostoma* spp. em cerca 48 horas após a captura. Para testar o seu antagonismo no ambiente foi utilizado um sistema prático e economicamente viável de produção massal de inóculo fúngico em grãos de arroz branco e grãos de milho moído. O fungo foi incorporado ao solo colonizado em grãos de milho moído tendo como parâmetro a concentração de 10.000 clamidósporos/g de solo, efetiva em ensaios *in vitro* preliminares. Tal abordagem provavelmente favoreceu o estabelecimento do antagonista, havendo redução significativa da população do nematóide alvo em solo não tratado sob condições semi-naturais, mostrando o potencial regulador deste agente biológico.

ABSTRACT

MACIEL, Alessandro Spalenza, D. Sc., Universidade Federal de Viçosa, July of 2009. **Nematode-trapping fungi in the control of dog's hookworms.** Adviser: Jackson Victor de Araújo. Co-Advisers: Artur Kanadani Campos, Laércio dos Anjos Benjamin and Leandro Grassi de Freitas.

The nematodes of the genus *Ancylostoma* are cosmopolitan endoparasites of dogs that cause various diseases in these animals and also are zoonotic geohelminths that can infect humans from soil. The control of the adult stage of these nematodes is based on the use of anti-helminthic; however, the use of biocontrol agents may be an additional measure reducing the population of pre-parasitic stages in development in the soil. This is justified by the fact that stray dogs parasitized are primarily responsible for environmental infestation with hookworm eggs transmitted in faeces. Among the biocontrol organisms, it is known that nematode-trapping fungi have been effective against nematodes of domestic animals. In this context, the objective of this study was to test various fungi isolated of predacious fungi, selecting the most infective for use in the control of infective larval form (L_3) of *Ancylostoma* spp. in soil under semi-natural conditions. The *Duddingtonia flagrans* fungus (Isolate CG768) was considered the most infective due to the reduction in the recovery of the target nematode L_3 and by the characteristic of producing many spores of resistance that can survive in soil, prolonging the life of the fungus in the absence of prey. The infectivity of this isolate could be observed in scanning electron micrographs that showed the destruction of L_3 of *Ancylostoma* spp. in about 48 hours after capture. To test the antagonism in the environment it was used a practical and economically viable system of mass production of fungal inoculum in grains of white rice and milled maize. The fungus was incorporated into the soil colonized in milled maize, taking as parameter the concentration of 10,000 chlamydospores/g of soil, previously effective *in vitro* tests. This approach probably favored the establishment of the antagonist with significant reduction of the target nematode population in untreated soil under semi-natural conditions, showing the potential regulator of this biological agent.

INTRODUÇÃO GERAL

O potencial papel dos animais de companhia de reservatório para zoonoses tem sido reconhecido como um problema de saúde pública no mundo (Schantz, 1994). Eles estão expostos a numerosos microorganismos tais como bactérias, vírus, rickétsias, micoplasmas, clamídias, protozoários, fungos e parasitos (Plaut et al., 1996; Geffray, 1999; Macpherson, 2005). Dentre estes animais, o cão (*Canis familiaris*) é o que mais convive com o ser humano (Leite et al., 2004), e historicamente, foi a primeira espécie domesticada cujo relacionamento começou em tempos pré-históricos, cerca de 12.000-15.000 anos atrás (Morey, 1994).

Este animal é um importante veiculador ambiental de parasitos (Traub et al., 2007) e sua população tem crescido consideravelmente nas zonas urbanas devido à procriação descontrolada (Dutta, 2002). Tal fato junto com a falta de saneamento e o crescimento desordenado das cidades em países em desenvolvimento aumenta os riscos de transmissão de zoonoses devido à proximidade com os seres humanos (Robertson et al., 2000). Segundo Macpherson et al. (2005) a população mundial de cães é estimada em mais de 500 milhões de animais. Entretanto, em 2003 a Organização Mundial da Saúde (OMS) já estimava em 600 milhões o número de cães em todo o mundo, dos quais 80% eram animais de rua abandonados, projetando o dobro desse número para 2013 (Martins, 2003). No Brasil a proporção homem:cão varia de 3:1 a 9:1 (Serafini et al., 2008), o que é preocupante uma vez que a OMS considera em 10% a percentagem máxima de cães em relação à população humana que não represente um fator de risco (Bâgel et al., 1990).

Os cães são hospedeiros de inúmeros parasitos e estão envolvidos na transmissão involuntária de mais de 60 infecções zoonóticas (Macpherson et al., 2000). Dentre elas, as parasitoses gastrintestinais, causadas por geohelmintos, estão entre as mais importantes e prevalentes infecções parasitárias destes animais (Silva et al., 1991; Blagburn et al., 1996). Uma vez que o solo de espaços públicos é freqüentemente infestado por ovos de geohelmintos veiculados nas fezes de cães, ele se torna um foco potencial de transmissão destes enteroparasitos (Santarém et al., 2004; Blazius et al., 2005; Macpherson, 2005). Epidemiologicamente, os cães errantes são os principais responsáveis pela contaminação do solo com ovos de helmintos (Ragozo et al.,

2002; Scaini et al., 2003; Santarém et al., 2004) uma vez que uma maior prevalência de parasitismo é observada nestes animais em comparação aos domiciliados, em virtude de não receberem tratamento antiparasitário e à facilidade com que circulam por áreas públicas (Alves et al., 2005; Palmer et al., 2007). Isto aumenta consideravelmente o risco de transmissão desses helmintos via solo para a população humana e animal (Corrêa e Moreira, 1995; Robertson et al., 2000; Labruna et al., 2006).

Os principais helmintos de interesse médico e veterinário pertencem ao Filo Nemathelminthes, que compreende os nematóides, e ao Filo Platyhelminthes, formado por cestóides e trematodes sendo que os cães podem ser parasitados por aproximadamente 17 espécies de trematodes, 17 de cestóides e 20 de nematóides (Soulsby, 1982). O nematóide que tem requerido grande atenção pelo seu potencial zoonótico, que tem o cão como hospedeiro definitivo, é do gênero *Ancylostoma* (Robertson et al., 2000; Miranda et al., 2008a; Katagiri e Oliveira-Sequeira, 2008). As espécies *Ancylostoma braziliense* Gomez de Faria (1910), *A. caninum* Ercolani (1859) e *A. ceylanicum* Looss (1911) são as mais prevalentes nestes animais (Soulsby, 1982; Baker et al., 1989; Traub et al., 2004) sendo importantes do ponto de vista veterinário e de saúde pública (Prociv, 1998; Robertson et al., 2000; Schantz, 2002). Segundo Urquhart et al. (1998) os ancilostomídeos são responsáveis por ampla morbidade e mortalidade em cães sendo que a espécie *A. caninum* é a mais patogênica devido à maior espoliação sanguínea (Soulsby, 1982; Burrows et al., 1995; Bowman et al., 2003).

O parasitismo intestinal por ancilostomídeos afeta cães de todas as idades sendo que a doença é comum nos animais com menos de um ano de idade, uma vez que nos adultos o desenvolvimento gradual de resistência etária torna menos provável o aparecimento de sintomas clínicos (Soulsby, 1982). Não há o desenvolvimento de uma imunidade efetiva (Boag et al., 2003; Blazius et al., 2005), por isso os cães são suscetíveis à infecção com ancilostomídeos ao longo da vida, mas a maior prevalência e o maior nível de infecção tendem a ser maiores em filhotes devido a um sistema imune imaturo em adição ao fato de que a principal via de infecção ser a lactogênica (Kalkofen, 1987; Ramírez-Barrios et al., 2004). Em cães filhotes, idosos e adultos imunossuprimidos podem ocorrer severos sinais clínicos como gastrenterites, obstrução intestinal, prolapso retal, abscessos, lesões

intestinais, hemorragias, anemia moderada ou severa, afecções respiratórias, hipoproteinemia, diarréia com sangue, além de emagrecimento e retardo no desenvolvimento, podendo evoluir para caquexia e morte (Georgi e Georgi 1989; Urquhart et al., 1998). Diferentemente, os cães adultos apresentam uma prolongada pré-patência e uma curta patência caracterizada por ser uma doença subclínica crônica (Reinemeyer, 1995). Em humanos, a infecção por ancilostomídeos de cães pode causar a síndrome da dermatite linear serpiginosa, mais conhecida como larva migrans cutânea (Morrison, 2001; Brenner e Patel, 2003), enterite eosinofílica (Dowd et al., 1994; Loukas et al., 1994), síndrome de Löffler's (Waldamez e Lizama, 1995; Schaub et al., 2002) e neurorretinite subaguda unilateral difusa (Venkatesh et al., 2005; Vedantham et al., 2006).

Os ancilostomídeos estão amplamente distribuídos geograficamente nas regiões tropicais e subtropicais, cujo solo retém umidade e está submetido a temperaturas mais elevadas (Chan, 1997) que são importantes para o embrionamento dos ovos e desenvolvimento e sobrevivência das larvas (Soulsby, 1982). Segundo Vinha (1965) o solo pode ser considerado um "hospedeiro intermediário" de geohelmintos dando condições para o desenvolvimento dos estágios não-infectantes e albergando a forma infectante para transmiti-la ao ser humano. O ciclo destes parasitos no solo tem início quando a larva rabditóide de primeiro estágio (L_1) é liberada do ovo após 24 horas de embrionamento sob condições adequadas (Ribeiro, 2004). É relevante ressaltar que diariamente uma fêmea de *A. caninum* pode ovipor uma média de 16.000 ovos, enquanto que uma de *A. braziliense* pode ovipor 4.000 ovos (Freitas, 1982). Esta larva de primeiro estágio se alimenta de bactérias e em 3 dias muda para larva rabditóide de segundo estágio (L_2), que também se alimenta, crescendo e mudando para larva filarióide de terceiro estágio (L_3), que é infectante (Soulby, 1982). A L_3 é impedida de se alimentar devido à permanência da cutícula do estágio anterior, permeável apenas por gases (Velho et al., 2003), que a protege e por isso pode sobreviver e permanecer infectiva por vários meses em um solo quente e úmido, ao abrigo da luz solar direta e da desidratação (Heukelbach e Feldmeier, 2008).

Os hospedeiros definitivos podem se infectar pelas vias oral, percutânea, transplacentária e lactogênica sendo a via oral a mais comum (Soulsby, 1982; Urquhart et al., 1998; Fortes, 2004). Na infecção oral, as larvas que não

migraram sistemicamente penetram nas glândulas gástricas ou nas glândulas de Lieberkühn do intestino delgado e se desenvolvem em larvas de quarto estágio (L_4). Posteriormente, elas voltam ao lúmen intestinal onde terminam o seu desenvolvimento, mudando para o estágio adulto após fixar-se na mucosa intestinal para realizar a hematofagia e a cópula, atingindo a maturidade 15 a 26 dias após a infecção, mas a espécie *A. caninum* pode atingi-la em 14 dias (Georgi e Georgi, 1989; Reinemeyer, 1995). O helminto adulto no cão pode viver uma média de 6 meses (Soulsby, 1982), no entanto, este período de vida pode se estender até dois anos para a espécie *A. caninum* (Fortes, 2004). Quando a infecção é percutânea, as L_3 penetram a pele ou a mucosa oral e atingem os capilares sangüíneos ou linfáticos de forma ativa podendo chegar ao coração e aos pulmões, carreadas passivamente pelo sangue do sistema venoso e dos ductos torácicos. No pulmão, as larvas penetram nos alvéolos pulmonares, permanecendo por 48 horas, e migram como L_4 para os bronquíolos, brônquios e traquéia chegando à faringe por expectoração (Fortes, 2004) e por deglutição atingem o intestino delgado onde se desenvolvem em vermes adultos imaturos ao sexto dia pós-infecção atingindo maturidade sexual ao 17º dia (Soulsby, 1982; Mittra et al., 1984). Segundo Velho et al. (2003) a infecção percutânea se deve ao tropismo das L_3 pela alta concentração de dióxido de carbono do tecido epitelial animal e humano. As L_3 que seguiram uma rota migratória somática na infecção percutânea ficam em hipobiose no tecido muscular de cães sensibilizados por infecções anteriores sobrevivendo por até 240 dias, no caso da espécie *A. caninum* (Soulsby, 1982). Em cadelas elas são reativadas durante o estro e no final da gestação podendo auto-infectar endogenicamente a fêmea ou infectar a prole intra-uterinamente via placenta e lactogenicamente via glândula mamária (Soulsby, 1982; Fortes, 2004). As larvas quiescentes nas cadelas podem ser transmitidas aos recém nascidos por até três ninhadas e em cada ninhada ser transmitida por até 20 dias após o parto (Soulsby, 1982).

Diversos autores têm relatado por meio de técnicas coproparasitológicas a elevada prevalência de endoparasitos em cães em diferentes cidades do Brasil, especialmente para o gênero *Ancylostoma,* o que indica um risco potencial de contaminação do solo (Tabela 1).

Tabela 1 – Porcentagem de amostras de fezes de cães positivas para ovos de *Ancylostoma* spp. em diferentes cidades do Brasil

Cidade	Amostras	*Ancylostoma* spp.	Referência
Anápolis, GO	66*	47	Francisco et al. (2008)
Araçatuba, SP	314*	45,2	Farias et al. (1995)
Araçatuba, SP	401**	53,1	Táparo et al. (2006)
Araguaína, TO	175**	50,28	Santos et al. (2006)
Balneário Cassino, RS	237*	71,3	Scaini et al. (2003)
Botucatu, SP	152* e 119*	17,1 e 31,9	Oliveira-Siqueira et al. (2002)
Campo Grande, MS	74*	56,8	Araújo et al. (1999)
Campos dos Goytacazes, RJ	68**	44,12	Miranda et al. (2008b)
Cuiabá, MT	121*	31,40	Almeida et al. (2007)
Curitiba, PR	264**	29,2	Leite et al. (2004)
Goiânia, GO	50* e 384**	22 e 9,9	Alves et al. (2005)
Guarulhos, SP	166**	10,8	Santos e Castro (2006)
Ilhéus, BA	150**	17,1	Magalhães et al. (2006)
Itabuna, BA	119*	47,9	Campos Filho et al. (2008)
Itapema, SC	158*	70,9	Blazius et al. (2005)
Itaperuna, RJ	77* e 97**	79,22 e 40,20	Silva et al. (2006)
Itaqui/Uruguaiana, RS	36*/36*	55,5/33,34	Moro et al. (2008)
Lages, SC	253**	21,32	Souza et al. (2006)
Lavras, MG	174**	58	Guimarães et al. (2005)
Londrina, PR	889**	39,8	Guimarães Júnior et al. (1996)
Monte Negro, RO	95**	73,7	Labruna et al. (2006)
Porto Alegre, RS	316*	79,1	Hoffmann et al. (1990)
Porto Alegre, RS	173**	42	Castro et al. (2001)
Praia Grande, SP	257*	45,9	Castro et al. (2005)
Ribeirão Preto	331*	41,7	Capuano e Rocha (2006)
Rio de Janeiro, RJ	204**	34,8	Vasconcellos et al. (2006)
Rio de Janeiro e Niterói, RJ	212**	20,28	Brener et al. (2005)
Santa Maria, RS	240**	69,6	Silva et al. (2007)
Santos, SP	150*	51,33	Jesus et al. (2006)
São Paulo, SP	903**	53,3	Fenerich et al. (1972)
São Paulo, SP	9.150*	59,8	Côrtes et al. (1988)
São Paulo, SP	1755**	12,7	Funada et al. (2007)
São Paulo, SP	353*	20,4	Gennari et al. (1999)
Uberlândia, MG	11.563**	61,2	Oliveira et al. (1990)
Uberlândia, MG	142**	57,02	Milken et al. (2007)
Viçosa, MG	437**	49,6	Araújo et al. (1986)

*Fezes coletadas de áreas públicas
**Fezes coletadas de cães domiciliados

A maioria dos nematóides não é parasita, no entanto algumas espécies são parasitas de plantas ou animais (Bekal e Becker, 2000). Os nematóides parasitas gastrintestinais de animais têm seu ciclo de vida dividido em duas fases: endoparasitária e de vida livre (ovos e larvas). Muitas vezes o parasitismo não se desenvolve com a infecção porque o agente é impedido de se estabelecer ou permanece em latência frente aos mecanismos de defesa do sistema imunológico do hospedeiro (Gronvold et al., 1996a), mas este equilíbrio pode ser comprometido por fatores como o clima, o nível nutricional, a raça, a

idade e o estado fisiológico dos animais (Coop e Kyriazakis, 1999). Epidemiologicamente a fase de vida livre ou ambiental do ciclo de vida é a mais importante por garantir a disseminação e perpetuação da nova geração do parasito com a infecção de mais hospedeiros (Soulsby, 1982; Neves, 2005). Em contrapartida, pelo fato de se desenvolverem no solo e habitá-lo até encontrar um hospedeiro, estas formas de vida de livre ficam vulneráveis não só a fatores abióticos, como condições meteorológicas, ou fatores bióticos, como alimento (quantidade e qualidade) e competições interespecíficas ou intraespecíficas, mas também a organismos vivos que naturalmente ocorrem no ambiente (Gronvold et al., 1996a; Stromberg, 1997) e são tidos como agentes de controle biológico (Kerry, 1987). Na ausência de inimigos naturais a população de vida livre de um parasita de animais poderia aumentar indiscriminadamente (Gronvold et al., 1996a; Delfosse, 2005).

No solo, diversos organismos como protozoários (Sayre, 1971; Canning, 1973), amebas, turbelários, tardígrados (Sayre e Wergin, 1979), copépodes (Lehman e Reid, 1993), colêmbolas (Gilmore e Potter, 1993; Lee e Widden, 1996), ácaros (Lysek, 1963; Imbriani e Mankau, 1983), nematóides predadores (Stirling, 1991), oligoquetas (Gronvold et al., 1996a), bactérias ,(Sayre, 1986; Stirling, 1988; Larsen, 1999) e fungos (Barron, 1977; Nordbring-Hertz, 1988) podem ser antagonistas das formas pré-parasitárias de nematóides. Destes microrganismos antagonistas da microflora do solo, os fungos têm grande potencial como agentes de biocontrole de nematóides de animais (Larsen, 2000). Aproximadamente 75% dos antagonistas de nematóides são fungos nematófagos (Van Gundy, 1985; Nordbring-Hertz, 1988), existindo mais de 200 espécies que utilizam hifas vegetativas modificadas morfologicamente ao longo do micélio para a captura e infecção de nematóides no solo (Li et al., 2000).

Os fungos nematófagos estão amplamente distribuídos geograficamente pelo mundo em diversos climas, habitando todos os tipos de solo, especialmente os que são ricos em matéria orgânica (Barron, 1977; Gray, 1987; Nordbring-Hertz et al., 2006). As pesquisas mostram que no campo sob condições experimentais ou naturais, espécies de fungos nematófagos são bons agentes de controle biológico de nematóides de animais (Larsen, 1999), uma vez que influenciam negativamente a transmissão destes parasitas (Ciarmela et al., 2002). Estes microrganismos são isolados a partir do solo e de fezes novas ou velhas de animais (Gronvold et al., 1993; Larsen et al., 1994;

Mahoney e Strongman, 1994; Hay et al., 1997). Tradicionalmente os fungos nematófagos são divididos em quatro grupos, de acordo com o seu modo de parasitismo sobre nematóides: predadores, que utilizam armadilhas adesivas ou hifais mecânicas; endoparasitas, que utilizam esporos; parasitas de ovos e cistos ou de fêmeas de fitonematóides, que utilizam apressórios; e produtores de toxinas, que paralisam os nematóides antes da invasão (Li et al., 2000; Nordbring-Hertz, 2004; Liu et al., 2009). Muitos dos fungos predadores e parasitas de ovos sobrevivem no solo utilizando os nematóides como uma fonte de nutrientes complementar a uma existência saprofítica, ao contrário dos endoparasitas que são na sua maioria parasitas obrigatórios (Nordbring-Hertz et al., 2006; De e Sanyal, 2009). Uma vantagem dos predadores e parasitas de ovos é que eles são fáceis de serem produzidos em laboratório (Jansson e Nordbring-Hertz, 1980).

Os fungos nematófagos, em sua maioria, são considerados mitospóricos, classificados como Deuteromycetes, classe Hyphomycetes, ordem Hyphomycetales e família Moliniaceae, apresentando micélio septado e bem desenvolvido, reproduzindo-se agamicamente por espóros exógenos, que são formados sobre ramificações das hifas (Van Oorschot, 1985; Barnett e Hunter, 1998). Estágios de reprodução sexuada destes fungos foram observados para algumas espécies que estão sendo reconhecidas como pertencentes ao filo Ascomycota (Griffin, 1994; Pfister, 1997). Em classificações mais recentes os fungos predadores mais comuns são considerados pertencentes à família Orbiliaceae, do filo Ascomycota (Yang et al., 2007; Liu et al., 2009).

A maioria das espécies de fungos nematófagos é predadora (Larsen, 1999) e se caracteriza por desenvolver um extenso sistema de hifas vegetativas ao longo das quais ocorre diferenciação morfológica em estruturas funcionais denominadas armadilhas que capturam e retêm nematóides vivos para poder infectá-los e nutrir-se de seu conteúdo interno (Barron, 1977). Tal mudança de uma fase saprofítica para uma fase parasitária com diferenciação morfológica em armadilhas e utilização de nematóides como alimento, influenciada por fatores bióticos e abióticos, provê uma vantagem nutricional para estes microrganismos no solo (Nordbring-Hertz, 1988; Nordbring-Hertz et al., 2006). Segundo Gray (1987) os fungos predadores diferenciam suas hifas vegetativas em seis estruturas de captura: hifas adesivas não modificadas ou

não diferenciadas; ramos adesivos; redes adesivas (bidimensionais ou tridimensionais); botões adesivos; anéis constritores e anéis não constritores. As armadilhas mais comuns são as redes tridimensionais, formadas por espécies do gênero *Arthrobotrys*, *Duddingtonia* e *Monacrosporium* (Gray, 1987) e são originadas de um ramo lateral que dá voltas anastomosadas com a hifa de origem formando uma rede pegajosa (Bird e Herd, 1995).

É possível que a interação antagônica entre estes organismos já ocorra naturalmente a milhões de anos devido à descoberta no México de um fóssil do nematóide *Oligaphelenchoides atrebora* parasitado por um fungo nematófago com aproximadamente 22,5-26 milhões de anos (Jansson e Poinar, 1986). Contudo, descobriu-se que o fungo predador *Orbilia fimicola* foi o primeiro derivado de seu ancestral Ascomiceto (não predador) a mais de 900 milhões de anos atrás (Padovan et al., 2005), sendo muito mais antigo do que o fóssil registrado. Esta relação predador/presa entre fungos e nematóides é conhecida cientificamente a mais de cento e vinte anos, em 1888, quando pela primeira vez na história Zopf relatou que nematóides ativos podiam ser capturados, infectados, mortos e digeridos pelo fungo *Arthrobotrys oligospora,* que foi isolado e descrito por Fresenius em 1852 (Barron, 1977). Em 1933, Drechsler mostrou que as armadilhas deste fungo possuíam um poderoso adesivo que auxiliava na fixação da presa (Barron, 1977). No entanto, observações da habilidade de fungos em destruir nematóides foram relatadas por Lohde em 1874 para o fungo endoparasita *Harposporium anguillullae* Zopf (Kerry, 1984; Gray, 1988), e por Kuhn em 1877 para um fungo descrito apenas em 1881 como *Tarichum auxiliare* (Gray, 1987). Posteriormente diversas espécies de fungos nematófagos foram isoladas e descritas (Gray, 1987).

O progresso no biocontrole usando fungos nematófagos em recentes anos tem sido notável (Akhtar e Malik, 2000). O uso de biocontroladores teve início com a crise dos nematicidas na década de 70 uma vez que não reduziam as populações de nematóides, apresentavam elevado custo, causavam problemas ao ambiente e à microbiota do solo, contaminavam lençóis freáticos e intoxicavam plantas e animais (Mankau, 1981; Thompson, 1987). Já as pesquisas envolvendo estratégias alternativas sustentáveis de controle de nematóides parasitos de animais com o uso de fungos nematófagos foram conseqüência da preocupação com a resistência às drogas anti-helmínticas, a degradação do ambiente e os resíduos nos alimentos (Waller e Larsen, 1993).

O interesse para fins de controle biológico de nematóides teve início quando Linford e Yap (1939) testaram alguns fungos predadores no controle de *Meloidogyne* spp. em plantas de abacaxi no Havaí. Paralelamente no final dos anos 30 começaram os estudos pioneiros do antagonismo destes fungos predadores sobre nematóides parasitos de animais (Descazeaux, 1939; Descazeaux e Capelle, 1939; Deschiens, 1939ab; Roubaud e Descazeaux, 1939; Roubaud e Deschiens, 1939). Já os primeiros estudos sobre biocontrole de ancilostomídeos foram realizados em 1958 por Soprunov (1966) que verificou significativa redução na infecção de mineradores russos, ao espalhar de 100 a 150g de esporos/m^2 de fungos predadores no interior de minas de carvão. No Brasil, o primeiro relato de fungos parasitando nematóides foi feito por Freire e Bridge (1985) que observaram ovos, juvenis e fêmeas de *Meloidogyne incognita* parasitados por *Paecilomyces lilacinus* e *Verticillium chlamydosporium* (sin. *Pochonia chlamydosporia*). No caso de parasitos de animais os estudos brasileiros pioneiros foram realizados por Araújo e colaboradores (Araújo et al., 1992; 1993) que reportaram que isolados do gênero *Arthrobotrys* e a espécie *Monacrosporium ellipsosporum* foram eficazes no controle de larvas de *Haemonchus placei*.

De acordo com Stirling (1991), os fungos predadores exibem uma grande diversidade quanto ao seu requerimento nutricional, sendo, portanto, algumas espécies certamente mais eficientes que outras como agentes de controle biológico. Da mesma maneira, a variabilidade é freqüentemente observada em cultura quando isolados de um mesmo táxon diferem marcantemente em cor, grau de esporulação e taxa de crescimento. Essa variação reflete diferenças genéticas, as quais também podem se manifestar como diferenças na virulência. Tais diferenças indicam que o primeiro passo no programa de controle biológico é a seleção de isolados mais virulentos. Por isso, apesar do grande número de espécies de fungos nematófagos, a maioria dos estudos têm se concentrado em espécies predadoras pertencentes aos gêneros *Arthrobotrys*, *Duddingtonia* e *Monacrosporium* (Larsen, 2000). Dentre os fungos predadores, a espécie *Duddingtonia flagrans* tem sido a mais estudada, havendo consideráveis características que propiciam o seu uso como agente de controle biológico de parasitos de animais, destacando-se a produção de grande número de clamidósporos que resistem as condições adversas (Larsen, 1999; Faedo et al., 2000; Dimander et al., 2003a; Waghorn

et al., 2003; Terrill et al., 2004). As pesquisas com *D. flagrans* foram inicialmente empreendidas nos países escandinavos (Gronvold et al., 1993; Larsen, 2000), mas este fungo foi descrito pela primeira vez na Grã-Bretanha como *Trichothecium flagrans* por Duddington (1949) e, posteriormente, incluído no gênero monoespecífico *Duddingtonia* por Cooke (1969) e por fim no gênero *Arthrobotrys* como *A. flagrans* por Scholler et al. (1999), mas para manter a continuidade da literatura de controle biológico tem-se mantido *Duddingtonia* (Skipp et al., 2002). De acordo com Van Oorschot (1985), esta espécie é caracterizada pela produção de conídios na extremidade de conidióforos, com formato elíptico a ovóide, septo mediano e medindo 25-50 µm de comprimento por 10-15 µm de largura (Cooke e Godfrey, 1964), mas principalmente pela produção de numerosos clamidósporos intercalados às hifas vegetativas (Scholler e Rubner, 1994). Sua atividade nematófaga é garantida por meio de hifas adesivas e redes tridimensionais adesivas (Barron, 1977; Larsen, 2000), mas segundo Bogus et al. (2005) seus metabólitos podem prejudicar significativamente a motilidade de larvas de nematóides.

Geograficamente tem ampla distribuição mundial com relatos na Nova Zelândia (Skipp et al., 2002), Austrália (Larsen et al., 1994), Europa continental (Virat, 1977; Larsen et al., 1991), América do Norte (Mahoney e Strongman, 1994), América Central (Mendoza-de-Gives et al., 1998) e Ásia (Chandrawathani et al., 1998; Sanyal, 2000), podendo ser isolado de esterco bovino e eqüino, silagem e vegetação em decomposição (Larsen et al., 1991; Mahoney e Strongman, 1994; Skipp et al., 2002). Devido à capacidade dos clamidósporos de *D. flagrans* de suportar condições adversas, eles têm sido administrados na alimentação de bovinos, ovinos, caprinos e eqüinos por resistirem à passagem através trato gastrintestinal destes animais e posteriormente germinarem nas fezes, formando hifas e armadilhas (Peloille, 1991; Larsen et al., 1994; Wolstrup et al., 1994; Fernandez et al., 1997; Faedo et al., 1998; Skipp et al., 2002; Chartier e Pors, 2003; Nordbring-Hertz et al., 2006). Este procedimento tem sido utilizado com sucesso na Austrália (Larsen et al., 1994; Faedo et al., 1998), Dinamarca (Gronvold et al., 2000; Faedo et al., 2002), Holanda (Eysker et al., 2005), Lituânia (Sarkunas et al., 2000), Nova Zelândia (Waghorn et al., 2003), Suécia (Dimander et al., 2003a), França (Paraud et al., 2007), Estados Unidos (Terrill et al., 2004) e Brazil (Dias et al., 2007).

A administração oral regular de biocontroladores fúngicos não é uma opção prática para cães devido à atual inviabilidade de incorporá-los em rações ou outros alimentos. Tal procedimento seria desmerecido pela maioria dos proprietários de cães que esporadicamente ou raramente controlam as endoparasitoses de seus animais de estimação com vermífugos por desconhecimento ou displicência. No entanto, a vermifugação deveria ser feita continuamente, pois animais domiciliados também assumem importância na contaminação de locais públicos (Robertson et al., 2000; Labruna et al., 2006). Isto justifica a utilização de uma abordagem onde o inóculo de fungos nematófagos seja incorporado ao solo, o que deve ser feito juntamente com um substrato de crescimento fúngico para favorecer o seu estabelecimento. Tal abordagem também é reforçada pelo elevado número de cães errantes, que assumem grande importância na manutenção e disseminação de helmintos no ambiente devido à maior freqüência de parasitismo em conseqüência do abandono (Labruna et al., 2006). Além do que, ações governamentais, como a informação da população sobre os riscos de transmissão, o controle das zoonoses transmitidas por animais domésticos e o controle da população de cães errantes nas zonas urbanas, mediante captura e castração (Uga e Kataoka, 1995), bem como a restrição ao acesso de locais públicos tanto para animais domiciliados quanto errantes são praticamente inexistentes na maioria das cidades brasileiras, resultando em um aumento do risco de exposição às zoonoses transmitidas por estes animais (Oliveira-Siqueira et al., 2002).

O controle biológico com fungos nematófagos poderá ser uma forte arma no combate de parasitas de cães no solo num futuro próximo. Entretanto, ele deve ser empregado como parte de um programa integrado em complementação ao controle químico da forma endoparasitária em cães domiciliados e ao controle da população de cães abandonados, uma vez que reduz satisfatóriamente as formas pré-parasitárias de um nematóide-alvo no ambiente (Waller e Larsen, 1993; Thamsborg et al., 1999) dificultando o processo no qual a infestação do ambiente torna-se uma infecção no hospedeiro final (Hashmi e Connan, 1989). Diante desta perspectiva formulou-se a hipótese que fungos nematófagos predadores podem ser agentes de controle biológico de larvas infectantes de espécies do gênero *Ancylostoma* que parasitam cães reduzindo a população dessas formas pré-parasitárias no solo.

O presente trabalho teve como objetivos:

1. Avaliar o efeito dos fungos nematófagos predadores *Arthrobotrys cladodes*, *A. conoides*, *A. musiformis*, *A. oligospora*, *A. oviformis*, *A. robusta*, *Duddingtonia flagrans*, *Monacrosporium appendiculatum, M. sinense*, *M. thaumasium* e *Nematoctonus robustus* sobre larvas infectantes (L_3) de *Ancylostoma* spp., selecionando o mais infectivo;
2. Avaliar o efeito de diferentes concentrações do isolado mais infectivo sobre L_3 de *Ancylostoma* spp. em solo pasteurizado;
3. Registrar com micrografias de luz a interação entre o isolado mais infectivo e larvas infectantes de *Ancylostoma* spp.;
4. Registrar com elétron-micrografias de varredura os processos envolvidos na captura e destruição de L_3 de *Ancylostoma* spp. pelo isolado mais infectivo após o início da interação;
5. Avaliar o efeito do isolado mais infectivo sobre L_3 de *Ancylostoma* spp. em solo não tratado sob condições semi-naturais.

CAPÍTULO 1

Predation of *Ancylostoma* spp. dog infective larvae by nematophagous fungi in different conidial concentrations

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Abstract: In the present work, it was evaluated the *in vitro* effect of 12 isolates from the fungal species *Arthrobotrys*, *Duddingtonia*, *Nematoctonus* and *Monacrosporium* genera in different conidial concentrations on the capture of *Ancylostoma* spp. dog infective larvae (L_3), on 2% water-agar medium at 25 °C, at the end of a period of 7 days. The concentrations used for each nematophagous fungus were 1,000, 5,000, 10,000, 15,000 and 20,000 conidia/Petri dish plated with 1,000 *Ancylostoma* spp. L_3. All nematode-trapping fungi isolates tested reduced the averages of the uncaptured *Ancylostoma* spp. L_3 recovered, with the increase of the fungal inoculum concentration, in comparison to the fungus-free control ($p < 0.05$). The adhesive network producing species were better predators than the constricting ring or adhesive knob producing species. *Duddingtonia flagrans* (Isolate CG768) was the most effective, reducing the averages of the uncaptured *Ancylostoma* spp. L_3 recovered in 92.8%, 96.3%, 97.5%, 98.3% and 98.9%, respectively in five fungal inoculum concentrations established. Other effective nematophagous fungi were *Arthrobotrys robusta* (Isolate I31), which reduced the averages of the uncaptured *Ancylostoma* spp. L_3 recovered in 85.4%, 88.3%, 90.7%, 92.5% and 95.2%, and *Arthrobotrys oligospora* (Isolate A183), with reductions of 66.6%, 79.8%, 86.8%, 89.5% and 90.8%, respectively for both, in the five fungal inoculum concentrations established. No difference was found between Isolates A183 and I31 in the conidial concentrations of 15,000/Petri dish. *Nematoctonus robustus* (Isolate D1) and *Arthrobotrys bronchophaga* (Isolate AB) had the smallest percentages of reduction among the tested isolates and showed the lowest predacious activity. The isolates CG768, I31 and A183 were considered potential biological control agents of *Ancylostoma* spp. dog free-living stages, being directly influenced by the fungal inoculum concentration.
Keywords: Nematophagous fungi, *Ancylostoma* spp., dogs, biological control

1. Introduction

A significant growth of domestic and street dog population is observed today, which, associated with the reduction in physical space, especially in urban areas, has narrowed the contact between those animals and humans, increasing the possibility of zoonotic infections (Gennari et al., 1999; Scaini et al., 2003; Santarém et al., 2004; Labruna et al., 2006). The presence of infected

dogs in public places contributes to environmental contamination by hookworm eggs because their feces are easily mixed into the soil, remaining in the environment for a long time and significantly increasing the risk of transmission of these parasitic nematodes to human and canine population (Robertson et al., 2000; Gennari et al., 2001). The nematode species *Ancylostoma caninum* Ercolani (1859) and *A. braziliense* Faria (1910) are geohelminth enteroparasites of domestic and wild dogs (Soulsby, 1982; Ribeiro, 2004) and require special attention because they are zoonotic parasites that cause a public health problem (Robertson et al., 2000; Schantz, 2002).

Several microorganisms parasitize or prey upon nematodes, whose action is known as biological control, since they decrease the level of nematode free-living stages in the soil ecosystem (Stirling, 1991; Chen and Dickinson, 2004). Among these microorganisms, the nematophagous fungi *Arthrobotrys*, *Duddingtonia*, *Nematoctonus* and *Monacrosporium* genera are able to capture, kill and digest animal parasitic nematodes, serving as potential biological control agents (Alves et al., 2003; Dimander et al., 2003a; Melo et al., 2003; Araújo et al., 2004; Campos, 2006).

Nematophagous fungi can undergo a process of hyphal differentiation into adhesive trap structures. For all trap-forming fungi, this process can be induced by external stimuli, such as the presence of nematodes (Jansson and Nordbring-Hertz, 1980), substances derived or excreted by them (Nordbring-Hertz, 1988), in adverse conditions such as water and/or nutrient shortage (Balan and Lechevalier, 1972), or spontaneously in some species (Feder et al., 1960). According to Nordbring-Hertz et al. (2006), the nematode-trapping fungus *A. oligospora* can be induced to form traps by the presence of small peptides, such as the phenylalanyl valine, with high proportion of nonpolar and aromatic amino acids or their amino acid components in combination with low-nutrient conditions or nutrient shortage in both liquid and solid media.

The nematode predating process starts when the fungus attracts the nematodes with traps or organic and inorganic substances such as CO_2, ammonia (Barron, 1977) and sialic acid (Jansson and Nordbring-Hertz, 1984), and then captures them in the traps. After the capture, regardless of the trap type, the fungus penetrates the nematode and develops inside it, consumes its content and its vegetative and reproductive structures emerge on the surface (Mota et al., 2003).

This survey used nematophagous fungi isolates of *Arthrobotrys*, *Duddingtonia*, *Monacrosporium* and *Nematoctonus* genera to evaluate, under *in vitro* conditions, the trapping of *Ancylostoma* spp. dog infective larvae (L_3) as to the increasing fungal inoculum concentration, with the objective of exploiting them as biocontrol agents.

2. Material and methods

Twelve isolates of predatory nematophagous fungi obtained from Brazilian soil samples and samples of animal feces were used in this experiment: *Arthrobotrys cladodes* (Isolate CG719), *A. conoides* (Isolate I40), *A. musiformis* (Isolate A144), *A*. *oligospora* (Isolate A183), *A. oviformis* (Isolate A121B), *A. robusta* (Isolate I31), *A. bronchophaga* (Isolate AB) *Duddingtonia flagrans* (Isolate CG768), *Monacrosporium appendiculatum* (Isolate CGI), *M. sinense* (Isolate SF53), *M. thaumasium* (Isolate NF34A) and *Nematoctonus robustus* (Isolate D1). The identification was based on the direct observation of the morphological characteristics, as the conidial size and the morphology of conidiophores in micro-culture, and trapping organs on nematode-infected culture, mainly following the descriptions furnished by Cooke and Godfrey (1964), Cooke (1969), Van Oorschot (1985) and Liu and Zhang (1994).

The isolates were stored at 4 °C in the Parasitology Laboratory of the Department of Veterinary of the Universidade Federal de Viçosa – UFV, Brazil, in test tubes containing 2% corn-meal-agar (2% CMA). To induce fungal inoculum proliferation, culture disks of each isolate, with a 5 mm diameter, containing 2% CMA were transferred to 50 mm x 10 mm Petri dishes containing a 2% water-agar (2% WA) culture medium. After the mycelial growth of this new culture, a 5 mm diameter culture block was cut out from the colony and placed upside down on the V8-agar (200 mL of Campbell's V8 juice, 3 g of $CaCO_3$, 18 g of agar and 800 mL of distilled water) in a 90 mm x 15 mm Petri dish. The Petri dishes were kept at 25 °C in the dark for 10 days to induce fungal inoculum proliferation.

After the incubation period, 10 mL of distilled water were added to the grown fungal cultures to remove the mycelial fragments and conidia from the V8-agar surface, with the use of a brush. The suspension was transferred to a 50 mL polypropylene centrifuge tube with a lid. The conidial residue was then

washed with 5 mL of distilled water taken from the medium surface. The fungal suspension was filtered through gauze (4 layers) and collected in a 50 mL Griffin glass, to reduce the mycelial fragments and obtain only the conidial suspension. The fungal suspension was stirred for 2 min on a magnetic stirrer with 1 drop of dispersant Tween 80 to maximize the conidial separation. After the fungal suspension was homogenized, two aliquots of 10 mL were collected with a micropipette to fill the Neubauer hemacytometer chambers and estimate the conidial average, according to Alfenas and Mafia (2007).

The *Ancylostoma* spp. L_3 were obtained from fresh feces of naturally infected urban street dogs by vermiculite-coproculture kept at 26 °C for 10 days. At the end of the incubation period, *Ancylostoma* spp. L_3 were harvested using the modified Baermann techniques (Ueno and Gonçalves, 1998), in which they were concentrated by gravity in 5 mL vacutainer-like glass tubes connected to a funnel. After 12 h, the sediment containing *Ancylostoma* spp. L_3 was transferred to centrifuge tubes and washed by centrifugation and resuspension in distilled water, five times at 1,000 rpm for 5 min. The supernatant was disposed at the end of each centrifugation. The methodology described by Barçante et al. (2003) was used to filter and eliminate debris to obtain viable and active *Ancylostoma* spp. L_3 in a clean preparation. The *Ancylostoma* spp. L_3 suspension selected was homogenized and six aliquots of 10 mL were collected with a micropipette and placed upside down on a slide marked with longitudinal lines to facilitate the counting of the L_3 individuals. Each aliquot was covered with a glass coverslip after the addition of 10 mL of lugol's solution to kill *Ancylostoma* spp. L_3. Then, they were counted and identified under a light microscope with the magnification of 40x. The average of *Ancylostoma* L_3 aliquots was calculated, allowing the estimation of the average in the total suspension. Before used in the experiment, the motility of *Ancylostoma* spp. L_3 was checked by microscopical examination.

The experiment consisted of five treatments containing 1,000 L_3 and increasing amounts of fungal inoculum concentration: 1,000 (1:1), 5,000 (5:1), 10,000 (10:1), 15,000 (15:1) and 20,000 (20:1) conidia/*Ancylostoma* spp. L_3. The control treatment contained only 1,000 L_3. Each treatment was tested in 48 mm x 12 mm Petri dishes containing 5 mL of 2% WA, at 25 °C, in the dark, for 7 days. A standard volume totalizing 250 mL of *Ancylostoma* spp. L_3 and conidia was added to the center of each Petri dish. The Petri dishes were unsealed to

reduce the excessive moisture inside the lid, avoiding consequent L_3 migration by hydrotropism.

The interaction between the isolates and L_3 on the Petri dishes was observed daily with a light microscope (40x and 100x). At the end of 7 days, the uncaptured L_3 were harvested from the 2% WA culture medium by the modified Baermann techniques (Ueno and Gonçalves, 1998), in which they were concentrated by gravity in 5 mL vacutainer-like glass tubes connected to a funnel. After discarding 3 mL of water without larvae, two drops of lugol's solution were added in the remaining volume containing the sediment to kill the *Ancylostoma* spp. L_3. Then, this volume of 2 mL containing dead *Ancylostoma* spp. L_3 was transferred for Peter's counting slide and counted under light microscope (40x), according to Peters (1952). The interaction between the most effective isolate against *Ancylostoma* spp. L_3 was examined by scanning electron microscopy using the methodology described by Nordbring-Hertz (1983). The scanning electron micrographs were taken 12 h after the initial capture.

The experiment was arranged in a complete randomized design with six replications per treatment, each experimental plot consisting of a Petri dish. A comparison among the averages of the fungal treatments was carried out by the Duncan's test, as well as a comparison using the fungus-free control by the Dunnett's test, both at 5% significance level, using the Statistica software, version 7.0 (Statsoft, 2004).

3. Results

Table 1 shows the averages, standard deviation and reduction percentages of *Ancylostoma* spp. L_3 in Petri dishes with a 2% WA culture medium, treated with the fungal isolates, in comparison to fungus-free control. All nematode-trapping fungi isolates used in the *in vitro* test were able to capture *Ancylostoma* spp. L_3 and significantly reduced the average of the uncaptured L_3 recovered in comparison to the fungus-free control ($p < 0.05$), within the period of observation. In general, the highest and the lowest predatory activity were achieved by adding 20,000 and 1,000 conidia per Petri dish, respectively.

All isolates in the five concentrations were significantly different ($p <$

0.05) when compared to the fungus-free control. The interaction between the isolates and the conidial concentration was significant ($p < 0.05$). The comparison between the treatments showed a variation in the efficiency of the isolates in reducing *Ancylostoma* spp. L_3, according to the fungal inoculum concentration. The Isolates CG768, I31 and A183 showed a better capture and destruction results and consequently greater decrease in the average of the uncaptured L_3 recovered.

The Isolate CG768 showed a high individual predatory activity against *Ancylostoma* spp. L_3. This effect has a direct relation with the increase of the fungal inoculum concentration: 92.8%, 96.3%, 97.5%, 98.3% and 98.9% of reduction of uncaptured *Ancylostoma* spp. L_3, for 1,000, 5,000, 10,000, 15,000 or 20,000 conidia added per Petri dish, respectively (Fig. 1H). The Isolate I31 reduced the average of uncaptured *Ancylostoma* spp. L_3 recovered by 85.4%, 88.3%, 90.7%, 92.5% and 95.2%, also with increasing fungal inoculum concentration (Fig. 1F), followed by the Isolate A183, with reductions of 66.6%, 79.8%, 86.8%, 89.5% and 90.8% (Fig. 1D). However, there was no difference between the Isolates I31 and A183 in the concentration of 15,000 conidia (Table 1). The same occurred in the concentration of 20,000 conidia among the Isolates A183, CG719 and SF53 (Table 1). The lowest reduction occurred in treatments with the Isolates D1 and AB with the same reduction of the uncaptured *Ancylostoma* spp. L_3 recovered in all concentrations. In general, trap formations were much more pronounced in Isolate CG768 Petri dishes in response to the addition of *Ancylostoma* spp. L_3, while the Isolates D1 and AB were the poorest trap-inducers, producing very few traps towards the end of the observation period, even after an increase of the inoculum.

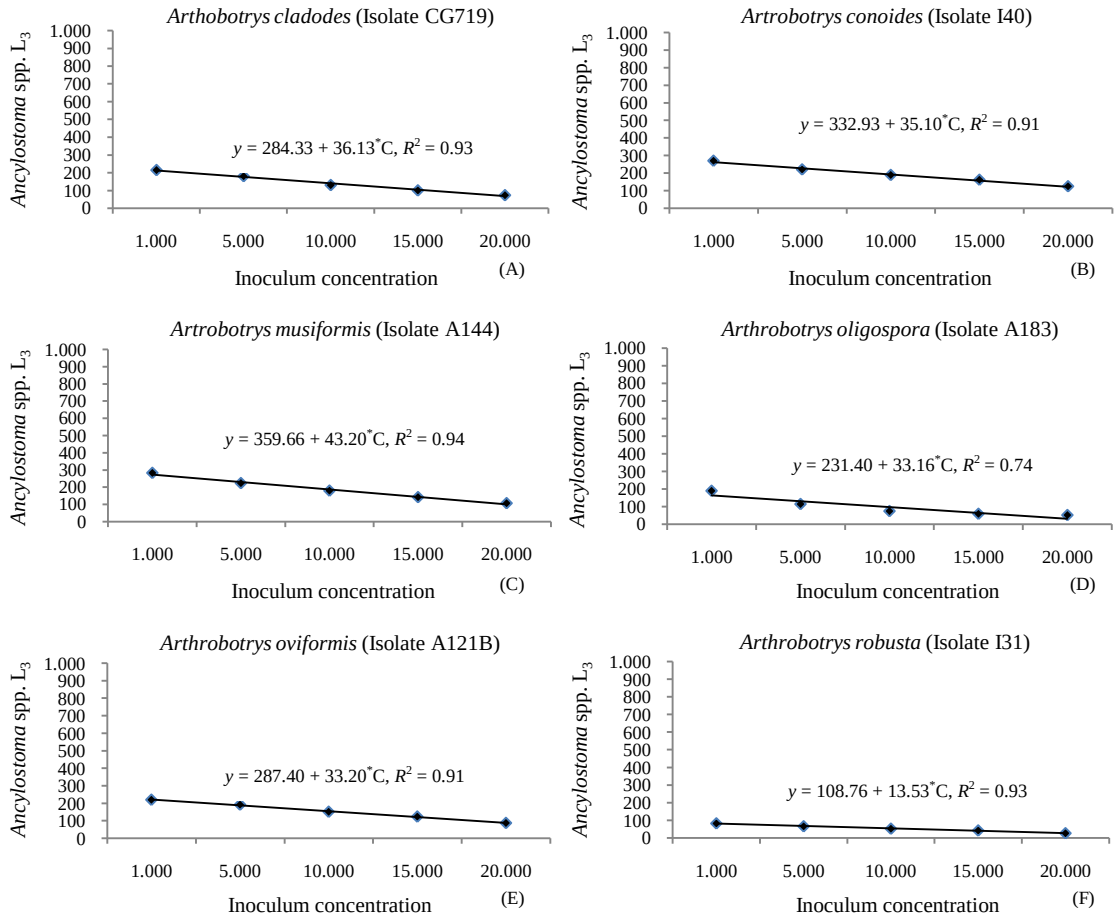
Table 1. Average values, standard deviation (±) and reduction percentage (%) of *Ancylostoma* spp. dog infective larvae recovered from 2% water-agar culture medium by the Baermann method after 7 days of interaction in Petri dishes containing fungal isolates *Arthrobotrys cladodes* (Isolate CG719), *A. conoides* (Isolate I40), *A. musiformis* (Isolate A144), *A. oligospora* (Isolate A183), *A. oviformis* (Isolate A121B), *A. robusta* (Isolate I31), *A. bronchophaga* (Isolate AB), *Duddingtonia flagrans* (Isolate CG768), *Monacrosporium appendiculatum* (Isolate CGI), *M. sinense* (Isolate SF53), *M. thaumasium* (Isolate NF34A) and *Nematoctonus robustus* (Isolate D1) in five fungal inoculum concentrations in comparison to fungus-free control.

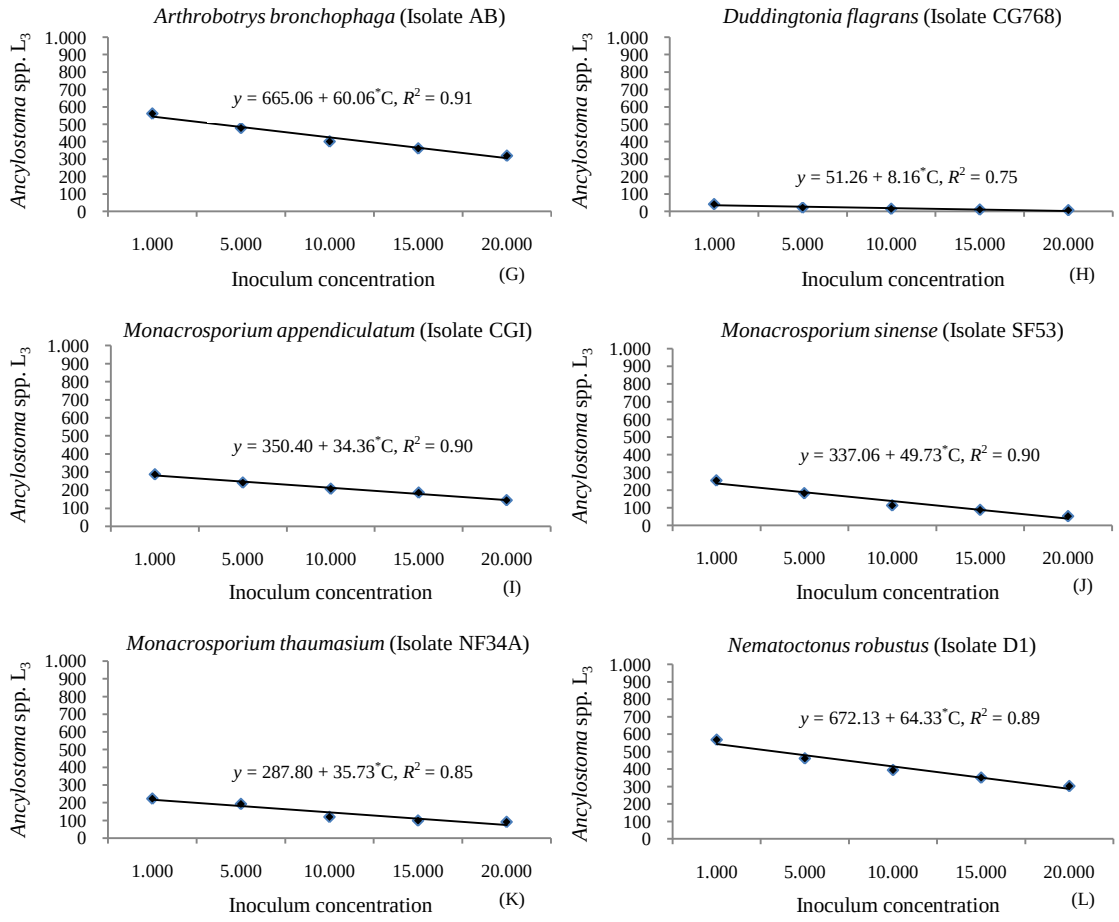
Fungal Isolates	Fungal inoculum concentrations (Conidia/Petri dish)				
	1,000	5,000	10,000	15,000	20,000
A. bronchophaga	561.66^{b} ± 28.57 (1.58)	479^{b} ± 28.39 (16.06)	401.66^{b} ± 25.62 (29.62)	361.66^{b} ± 8.89 (36.63)	320^{b} ± 26.07 (43.93)
A. cladodes	214.33^{e} ± 18.56 (62.44)	180.33^{d} ± 13.41 (68.40)	130.33^{f} ± 10.91 (77.16)	100.33^{f} ± 1.96 (82.42)	73.66^{fg} ± 14.71 (87.09)
A. conoides	269.66c^{d} ± 17.40 (52.75)	220^{c} ± 13.74 (61.45)	188.33^{cd} ± 10.83 (67)	161^{d} ± 6.78 (71.79)	123.66^{cd} ± 23.16 (78.33)
A. musiformis	282.33^{c} ± 21.66 (50.53)	223^{c} ± 15.98 (60.92)	179.33^{d} ± 11.57 (68.58)	141.66^{de} ± 11.27 (75.18)	106.66^{de} ± 14.12 (81.31)
A. oligospora	190.66^{f} ± 42 (66.59)	115.33^{e} ± 15.88 (79.79)	75.33^{g} ± 10.91 (86.80)	59.66^{g} ± 1.96 (89.55)	52.66^{g} ± 14.71 (90.77)
A. oviformis	219.33^{e} ± 19.08 (61.57)	191.66^{d} ± 5.98 (66.41)	151^{e} ± 11.64 (73.54)	123.66^{e} ± 8.98 (78.33)	87.33^{ef} ± 24.28 (84.70)
A. robusta	83.16^{g} ± 6.21 (85.43)	66.66^{f} ± 6.65 (88.32)	53^{h} ± 2.75 (90.71)	43^{g} ± 5.17 (92.46)	27.33^{h} ± 6.28 (95.21)
D. flagrans	41.33^{h} ± 11.07 (92.76)	21.33^{g} ± 3.5 (96.26)	14.33^{i} ± 1.5 (97.49)	9.66^{h} ± 0.81 (98.31)	6.33^{i} ± 1.96 (98.89)
N. robustus	568^{b} ± 30.59 (0.47)	460.66^{b} ± 25.75 (19.28)	393.66^{b} ± 21.92 (31.02)	350^{b} ± 9.79 (38.67)	301.66^{b} ± 44.69 (47.14)
M. appendiculatum	287.33^{c} ± 16.03 (49.65)	241.33c ± 19.33 (57.71)	207^{c} ± 10.86 (63.73)	185.66^{c} ± 5.57 (67.47)	143.33^{c} ± 25.38 (74.88)
M. sinense	254.33^{d} ± 29.48 (55.43)	182^{d} ± 24.09 (68.11)	113.66^{f} ± 13.47 (80.08)	88^{f} ± 9.87 (84.58)	52.66^{g} ± 12.62 (90.77)
M. thaumasium	222.66^{e} ± 25.03 (60.98)	192.66^{d} ± 13.06 (66.24)	119.33^{f} ± 14.78 (79.09)	98.66^{f} ± 10.63 (82.71)	91^{ef} ± 7.56 (84.05)
Fungus-Free control	570.67^{a} ± 24.38				

Average values followed by different superscripts in the same column are significantly different by the Duncan test ($p < 0.05$) and significantly different from the fungus-free control by the Dunnet test ($p < 0.05$). Inside the parentheses: Reduction percentage = 100 - (treatment average x 100/control group average).

The averages for the reduction of *Ancylostoma* spp. L_3 were used to estimate equations adjusted to the treatments with all the isolates. A high association degree (R^2 > 0.7) between the average of the uncaptured *Ancylostoma* spp. L_3 recovered and the fungal inoculum concentration was found with significant regression coefficients (p < 0.05), indicating a direct influence of the concentration on the reduction of uncaptured *Ancylostoma* spp. L_3 in the 2% WA culture medium (Fig. 1A-L).

A stronger reduction in the average of the uncaptured *Ancylostoma* spp. L_3 recovered from the 2% WA culture medium was observed, starting from the concentration of 1,000 conidia in the treatments with the Isolates CG768 and I31 (Fig. 1H and F), even in the lowest conidia concentration, resulting in a smaller slope angle. In the case of the Isolate CG768, the reduction was almost 100%, starting from the concentration of 5,000 conidia/Petri dish (Fig. 1H).





*Averages of the linear regression curve, statistically significant by the F-test ($p < 0.05$).

Fig. 1. Linear regression curves of the average of non-predated *Ancylostoma* spp. dog infective larvae (L_3) recovered from the 2% water-agar medium, initially containing 1,000 L_3, after 7 days of interaction, according to the increase of the fungal inoculum concentration in the treatments with the following fungal isolates: (A) *Arthrobotrys cladodes* (Isolate CG719); (B) *A. conoides* (Isolate I40); (C) *A. musiformis* (Isolate A144); (D) *A. oligospora* (Isolate A183); (E) *A. oviformis* (Isolate A121B); (F) *A. robusta* (Isolate I31); (G) *A. bronchophaga* (Isolate AB); (H) *Duddingtonia flagrans* (Isolate CG768); (I) *Monacrosporium appendiculatum* (Isolate CGI); (J) *M. sinense* (Isolate SF53); (K) *M. thaumasium* (Isolate NF34A); and (L) *Nematoctonus robustus* (Isolate D1).

Observations under light microscope (40x and 100x) showed that *Ancylostoma* spp. L_3, were captured and alive, after 12 h (Fig. 2H); after 24 h, they were captured and killed; and after 48 h, they were destroyed by the *D. flagrans* (Isolate CG768). On the first experimental day, the number of free *Ancylostoma* spp. L_3 in treatment Petri dishes was smaller than in the fungus-free control, with increasing difference on the other days. In addition, a decrease of these values was observed over the experimental period. *Ancylostoma* spp. L_3 exhibited the highest motility and migration in both fungal

treatments and fungus-free control, being widely dispersed over the WA 2% surface. In the fungus-free control group, some of them died without the presence of the predatory fungi, while others migrated to the periphery and tended to concentrate in the border of the WA 2% in Petri dishes.

The similarities, in terms of general morphology, nematophagous habits and conidial dimensions in this study are consistent with the published descriptions of the fungal species tested. In all treatments, the conidial production was observed around 2 days after the initial capture of *Ancylostoma* spp. L_3. However, the sporulation was higher between 4 and 6 days. During the experimental period, free *Ancylostoma* spp. L_3 were occasionally seen migrating freely, with traps and conidia attached to their cuticle, suggesting that they had succeeded in releasing themselves and evidencing the presence of adhesive substance on the conidia surface. These fungi were relatively easy to culture on artificial media with V8-agar. The conidia of the fungus *D. flagrans* were elliptical-shaped (Fig. 2A). The fungal colonization of the 2% WA medium and capture of *Ancylostoma* spp. L_3 were not observed in the fungus-free control (Fig. 2G). In the treatments with the fungus *D. flagrans* (Isolate CG768), the formation of abundant chlamydospores intercalating the hyphae (Fig. 2B) was observed on the sixth day. These chlamydospores had globular protuberances on their surface (Fig. 2C).

A scanning electron micrograph showed that the fungus *D. flagrans* (Isolate CG768) captured the *Ancylostoma* spp. L_3 by adhesive three-dimensional network traps, grown on the surface of the dialysis membrane (Fig. 2D, E and F). Two fungus-larvae interaction points were identified 12 h after the initial capture, with the presence of bacillus rod-shaped bacteria associated to fungal/nematode interaction sites in possible penetration points. In one of these points, the modified hyphae involved the *Ancylostoma* spp. L_3 (Fig. 2F).

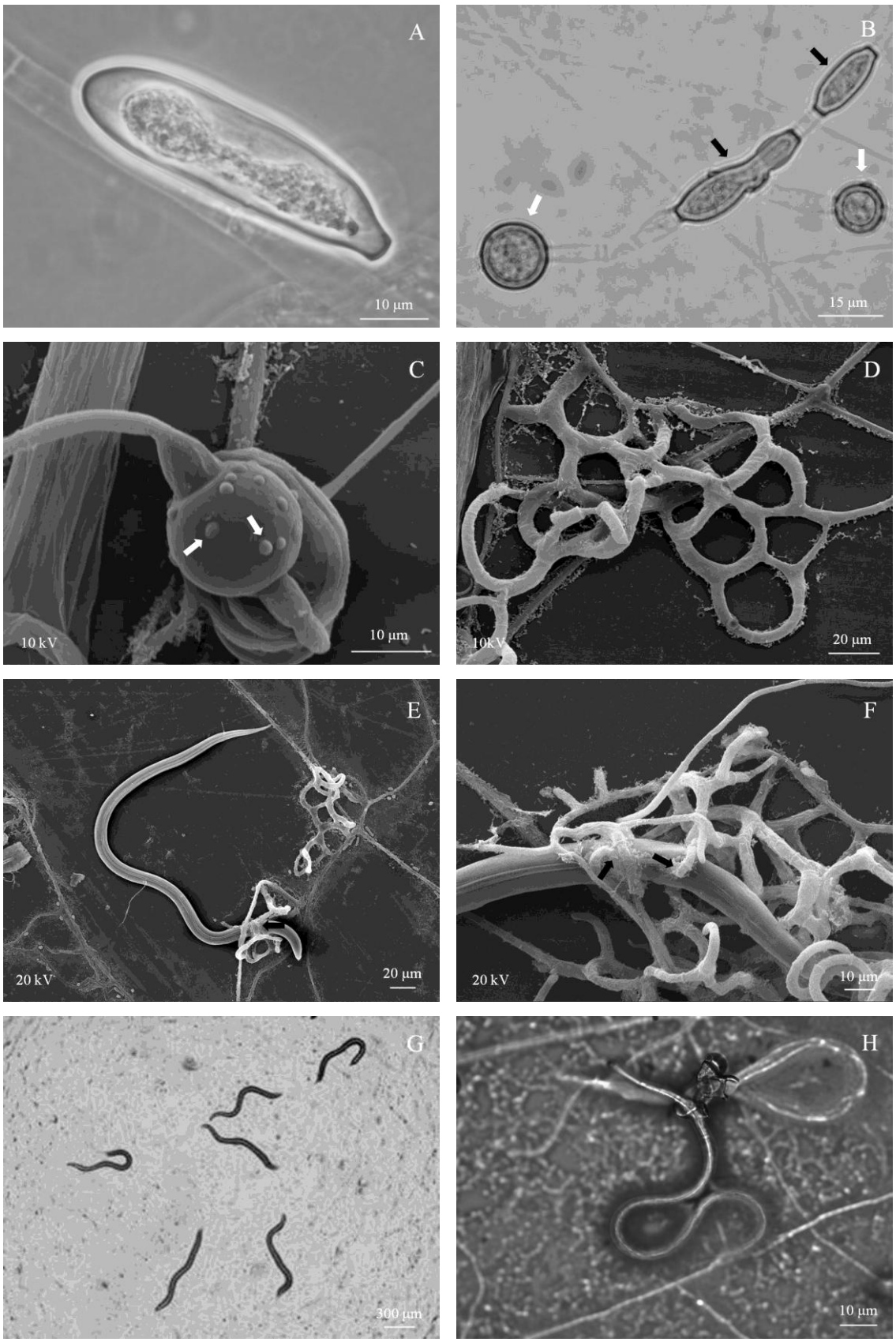


Fig. 2. (A) Light micrograph of a conidium obtained from the microculture of *D. flagrans* (Isolate CG768). (B) Light micrograph from mature chlamydospores (white arrow) and developing chlamydospores (black arrow), intercalated with the vegetative hyphae obtained from the micro-culture of *D. flagrans* (Isolate CG768). (C) Scanning electron micrograph of chlamydospore of *D. flagrans* (Isolate CG768) showing globular protuberances on the surface

(white arrows). (D) Scanning electron micrograph of adhesive three-dimensional network traps of *D. flagrans* (Isolate CG768). (E and F) Scanning electron micrograph of predated *Ancylostoma* spp. L_3, 12 h after the initial capture by three-dimensional network traps of *D. flagrans* (Isolate CG768), with fungus-larvae interaction points, which were possible penetration points and the presence of high amounts of Nematophagous Fungus Helper Bacteria (black arrows). (G) Light micrograph of non-predated *Ancylostoma* spp. L_3 in a 2% water-agar medium fungus-free control. (H) Light micrograph of *Ancylostoma* spp. L_3 captured alive, 12 h after the initial capture by *D. flagrans* (Isolate CG768) in a 2% water-agar medium.

4. Discussion

These results clearly show the susceptibility of *Ancylostoma* spp. L_3 to *Arthrobotrys*, *Duddingtonia*, *Monacrosporium* and *Nematoctonus* genera as well as the capacity of isolates of these nematophagous fungi tested to trap this enteroparasite nematode of dogs.

The trapping structures of *Arthrobotrys cladodes* (Isolate CG719), *A. conoides* (Isolate I40), *A. musiformis* (Isolate A144), *A. oligospora* (Isolate A183), *A. oviformis* (Isolate A121B), *A. robusta* (Isolate I31), *D. flagrans* (Isolate CG768), *Monacrosporium appendiculatum* (Isolate CGI), *M. sinense* (Isolate SF53) and *M. thaumasium* (Isolate NF34A) consisted of three-dimensional adhesive networks as described by Gray (1987). Constricting ring and adhesive knob traps were observed in Petri dishes containing *A. bronchophaga* (Isolate AB) and *N. robustus* (Isolate D1), respectively, and are consistent with the traps described for these species by Barron (1977). This trap formation was observed around 12 h after the beginning of the interaction between the microorganisms used. This process is in accordance with the reports of Pramer (1964), who observed a hyphal differentiation into nematode trap structures in the first hours of interaction. However, in the present study, the traps were originated directly from the germination of conidia and can be called conidial traps (Nordbring-Hertz et al., 2006). Such ability is a desirable characteristic for the application of these fungi as biocontrol agents of *Ancylostoma* spp. dog free-living stages because they can be dispersed in the environment, which increases the chances of trap. This developmental pattern occurs in almost all trap-forming species, when conidia are allowed to germinate in natural substrates (Persmark and Nordbring-Hertz, 1997). Besides, this trapping mechanism confers a significant ecological advantage, avoiding any fungistatic effects in the environment that may inhibit the normal germination of conidia (Gray, 1987).

Since traps can be generated directly from conidia, the observation of many free *Ancylostoma* spp. L_3 with conidia adhered on the cuticle suggests that this larva will be soon captured and destroyed.

In the current survey, although the Isolates CG768 and I31 showed high effectiveness in reducing the average of uncaptured *Ancylostoma* spp. L_3 recovered, it was lower than the effectiveness reported in a previous work by Maciel et al. (2006), in which these isolates showed greater efficiency in the capture of *Ancylostoma* spp. L_3 in the concentration of 1,000 conidia/Petri dish, at 25 °C for 10 days. The same work also tested *M. thaumasium* (Isolate NF34A) and found better results, which may be due to a
higher temperature and longer time of interaction among microorganisms. In the case of the fungus *D. flagrans*, it is known that it has slow growth rates at temperatures lower than 25 °C (Larsen, 1991) and optimum growth at 30 °C, producing more traps on agar (Gronvold et al., 1996b).

Others fungi tested, such as *A. musiformis* (Isolate A144) and *A. conoides* (Isolate I40) showed satisfactory results in the control of *Ancylostoma* spp. L_3. These species have already demonstrated to be efficient, under *in vitro* conditions, against *Ancylostoma* spp. dog free-living stages (Graminha et al., 2001). The lower efficiency observed for fungi *A. bronchophaga* (Isolate AB) and *N. robustus* (Isolate D1) in this work is probably linked to the mycelial growth speed, once the nematophagous fungi can be grouped as: fast-growing fungi, which are the adhesive network-forming; followed by the adhesive knob-forming; or slow-growing fungi, which are the constricting ring-forming (Cooke, 1964). The morphology of the trap structures can also explain the differences in the averages of the uncaptured *Ancylostoma* spp. L_3 recovered. The probable reason is that each nematophagous species produces a kind of trap that may interfere in the efficiency to control the nematodes attracted to the traps (Nordbring-Hertz, 1988). It is possible that, the constricting ring and adhesive knob are less efficient in nematode trapping because they take up a smaller area in the culture medium than the adhesive networks, with a single point of contact to hold the prey, having fewer chances to interact with the migrating nematode larvae in comparison to the adhesive networks. However, the morphological design of the network, with its adhesive layer, ensures that the captured prey becomes entangled and stuck to others points of contact of the network as it struggles (Gray, 1987). In addition, more than one nematode larva

can be trapped in the same network. This suggests that predatory fungi with adhesive networks would be able to capture and consume larger *Ancylostoma* spp. L_3 than fungi with constricting ring or adhesive knob.

A daily observation of the Petri dishes showed numerous *Ancylostoma* spp. L_3 captured at the cephalic portion, which may probably happen because this portion is the first part of the nematode body to get in contact with the traps when they are attracted by this structure. The carbohydrates on the surface are found scattered over the whole surface, but they are frequently found at the head or at the tail, which explains the frequent binding of fungal traps to such nematode region, as reported by Jansson (1987). It was known that the adhesive substances on the surface of the trap hyphae hold the nematode, although the carbohydrate-binding protein (lectin) found on the fungal traps, which specifically bind to the carbohydrate receptor found on the nematode cuticle, would probably be enough to hold the nematode (Zuckerman and Jansson, 1984; Jansson, 1987; Murray and Wharton, 1990). Other interesting observation was the presence of scattered rod-shaped bacteria on the cellulose membrane surface of the Petri dishes, mainly at the points of interaction between the fungus *D. flagrans* and *Ancylostoma* spp. L_3. These bacteria probably act as helpers of the fungus *D. flagrans* activity and, according to Dupponois et al. (1998), they were called Nematophagous Fungus Helper Bacteria (NHB) and may be involved in the predation, sporulation and pathogenicity systems of nematophagous fungi. Moreover, the same researcher reported that these bacteria produce substances that would act as molecular bridges between fungi and nematodes, which could explain the high amount of such bacteria at the interaction points 12 and 24 h after the beginning of the capture of *Ancylostoma* spp. L_3. Due to this fact, the study of the effect of these bacteria in association to the fungus *D. flagrans* could provide a better understanding of their function in the interaction process with *Ancylostoma* spp. L_3, since only Campos et al. (2008) observed this bacterial association with fungus *D. flagrans* in literature, although the researcher has worked with predation of *Haemonchus contortus* L_3. The association of bacteria with nematophagous fungi is also recorded by Graminha et al. (2001), who studied the pathogenicity of the fungi *Arthrobotrys musiformis* and *A. conoides* on the of *H. contortus* and *Ancylostoma* spp. L_3.

It is known that some nematophagous fungi may not need stimulation for

trap formation, but others may be stimulated to form the traps by the presence of the nematode, inductive substances and others in adverse conditions. It is possible that the presence of *Ancylostoma* spp. L_3, with its rapid movements, or substances derived or excreted by it, in the Petri dishes containing 2% WA, stimulated trap formation by the tested fungal isolates. This can be explained by the fact that a culture medium poor in nutrients, such as 2% WA, encourages the nematophagous fungi to use nematodes as an alternative source of available nutrients (Scholler and Rubner, 1994). Then, the formation of trap structures will be a symptom of nutritional deficiency in nematophagous fungi, since in a culture medium with substrates containing adequate carbon and nitrogen concentrations, these fungi live as saprophytes, forming few or no trap structures (Scholler and Rubner, 1994; Morgan et al., 1997). Barron (1977), discussing about the induction of trap formation, emphasizes that researchers studying morphogenesis in predatory fungi, usually confined their studies to a single predator, hampering comparisons because most studies use other species. In addition, different species or even different strains of the same species may behave differently in their response to both kind and concentration of morphogenic inductor, although many factors are involved in trap formation (Barron, 1977). In natural environments, the nutritional conditions are generally unfavorable. Then, not only the presence of the nematode prey, but also the lack of nutrients may favor the production of trapping devices (Balan and Lechevalier, 1972). In addition, there is a range of peptides or amino acids in the soil, resulting from the biological degradation of proteinaceous material, capable of eliciting trap formation (Nordbring-Hertz, 1973). Based on these previous statements and *in vitro* results of the present survey, it is probable that the trap formation and consequent capture of *Ancylostoma* spp. L_3 by the nematophagous fungi *D. flagrans* (Isolate CG 768), *A. robusta* (Isolate I31) and *A. oligospora* (Isolate A183) will be favored in the soil.

In the present study, *D. flagrans* (Isolate CG768) not only showed greater virulence against *Ancylostoma* spp. L_3, presenting the best results in comparison to the other fungi tested, but also produced abundant chlamydospores, as described for this species (Juarez and Mendoza-de-Gives, 1998; Larsen, 1999), which are thick-walled structures, developed from the hyphae, that appear in unfavorable growth conditions (Scholler and Rubner, 1994) and can originate hyphae, conidiophores and conidia (Barron, 1977).

Chlamydospores are persistent in the environment, which favors their establishment and survival, and consequently their use as a biological control agent (Faedo et al., 2000). This fungal species is the most studied nematophagous fungus in experiments throughout the world and is considered promising for the biological control of gastrointestinal nematodiosis of several species of domestic animals (Gronvold et al., 1996a; Larsen, 2000; Faedo et al., 2002; Fontenot et al., 2003). Then, these characteristics and statements show that *D. flagrans* (Isolate CG768) could be an alternative for the biological control of *Ancylostoma* spp. L_3.

The administration of anthelminthics to animals has been the method used to prevent environmental contamination by *Ancylostoma* spp. eggs and larvae. However, the nematophagous fungi could be used jointly in situations where the environment is already contaminated, offering an environmentally sustainable method of control. The researchers Waller and Larsen (1993) discuss that the fungal application in the biological control of free-living stages of parasitic nematodes can help the chemical control. This control should be used not only in the prediction of major environmental infestation by free-living forms of helminths but also when there are better conditions for the fungal growth in the environment, preventing clinical parasitism and allowing the survival of sufficient larvae to develop animal immunity. The application of the fungus as a biological control agent of *Ancylostoma* spp. dog free-living stages could be of great use in Brazil, since dog's hookworm larvae are widespread in the soils, public squares and public parks (Santarém et al., 2004). This applicability can be enhanced, since street dogs must be considered in a worm control program in urban centers (Labruna et al., 2006). Although these animals play an important role in the maintenance and dissemination of these parasites in the urban environment, they have been excluded from programs. Given the close interaction between dogs and humans, it becomes absolutely necessary to use an adequate control in order to reduce the environmental contamination by infective larvae and thus minimize the risk of human and canine infection.

Nematophagous fungi present different predatory activities. While some isolates need only a few conidia to get the expected effect, others need a large inoculum to get the same effect. The varying susceptibility of *Ancylostoma* spp. L_3 to the different fungal isolates suggests that *in vitro* studies are important to select nematophagous fungi before their application for biological control.

Although such tests usually overestimate the activity of a biological control agent by not allowing the nematode to escape or even by not reproducing the possible environmental changes and interferences commonly found in the soil, they have nevertheless the advantages of a limited physical space and shorter evaluation time (Gomes et al., 2001). To choose a fungal isolate, it is also necessary to consider field tests that can prove the efficiency of these microorganisms as potential biological control agents. Research should focus on the best way to implement these fungi in the environment, evaluating if the formulations consisting of vegetative, reproductive or resistance structures would allow their establishment in the complex soil ecosystem and enable the control of *Ancylostoma* spp. dog free-living stages, breaking the life cycle of this parasite. Results showed that most fungi isolates tested were efficient in the *in vitro* destruction of *Ancylostoma* spp. L_3 and are promising alternatives in the control of free-living stages nematodes in infested areas. However, further studies are necessary to evaluate if it could bring damage to the soil microfauna.

CAPÍTULO 2

Scanning electron microscopy of *Ancylostoma* spp. dog infective larvae captured and destroyed by the nematophagous fungus *Duddingtonia flagrans*

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Abstract: The interaction between the nematode-trapping fungus *Duddingtonia flagrans* (Isolate CG768) and *Ancylostoma* spp. dog infective larvae (L_3) was evaluated by means of scanning electron microscopy. Adhesive network trap formation was observed 6 h after the beginning of the interaction, and the capture of *Ancylostoma* spp. L_3 was observed 8 h after the inoculation these larvae on the cellulose membranes colonized by the fungus. Scanning electron micrographs were taken at 0, 12, 24, 36 and 48 h, where 0 is the time when *Ancylostoma* spp. L_3 was first captured by the fungus. Details of the capture structure formed by the fungus were described. Nematophagous Fungus Helper Bacteria (NHB) were found at interactions points between the *D. flagrans* and *Ancylostoma* spp. L_3. The cuticle penetration by the differentiated fungal hyphae with the exit of nematode internal contents was observed 36 h after the capture. *Ancylostoma* spp. L_3 were completely destroyed after 48 h of interaction with the fungus. The scanning electron microscopy technique was efficient on the study of this interaction, showing that the nematode-trapping fungus *D. flagrans* (Isolate CG768) is a potential exterminator of *Ancylostoma* spp. L_3.
Keywords: Nematophagous fungus, *Duddingtonia flagrans*, *Ancylostoma* spp., dogs, biological control

1. Introduction

Nematophagous fungi have been studied as an alternative for controlling several nematodes (Araújo et al., 1993; Nansen et al., 1996; Chandrawathani et al., 1998; Araújo and Guimarães, 2002; Oliveira et al., 2002; Castro et al., 2003).

The species *Duddingtonia flagrans* is an important nematode-trapping fungus that has been used as a biological control agent of the free-living stages of gastrointestinal parasites of domestic animals (Baudena et al., 2000; Paraud et al., 2005; Maciel et al., 2006; Rocha et al., 2007).

Since dog parasite helminths of the *Ancylostoma* genus have been requesting attention for their zoonotic potential (Soulsby, 1982; Schantz, 1991; Prociv and Croese, 1996; Bahgat et al., 1999; Robertson et al., 2000), the destruction of their infective stages by using nematophagous fungi may be an alternative for the control of animal parasitic nematodes under environmental conditions.

Thus, the characterization of the fungus x larvae interaction processes would be very important, once this interaction is beneficial to the nematophagous fungi but not to the nematode larvae. This could influence on the selection of promising fungal isolates which might be used in biological control programs (Mendoza-de-Gives, 1999). Despite the studies that have been carried out, there is little information on the ultrastructural aspects of the interaction between the fungus *D. flagrans* and the gastrointestinal parasite nematode infective larvae of domestic animals.

The scope of this study was to observe and understand the interaction processes between the nematode-trapping fungus *D. flagrans* (Isolate CG768) against *Ancylostoma* spp. dog infective larvae (L_3).

2. Materials and methods

2.1. Acquirement of *Ancylostoma* spp. infective larvae

The *Ancylostoma* spp. L_3 were obtained from fresh feces of naturally infected urban street dogs by means of vermiculite-coproculture kept for 10 days at 26 °C. At the end of the incubation period, the L_3 were extracted by the modified Baermann's method, as described by Ueno and Gonçalves (1998). After 12 h, the sediment containing *Ancylostoma* spp. L_3 was transferred to a centrifuge tube and washed with distilled water five times at 1,000 rpm for 5 min. The supernatant was disposed at the end of each centrifugation. The methodology described by Barçante et al. (2003) was used to filter and eliminate debris to obtain viable and active *Ancylostoma* spp. L_3 in a clean preparation.

The selected *Ancylostoma* spp. L_3 suspension was homogenized and six aliquots of 10 mL were collected with a micropipette and placed over a slide marked with longitudinal lines to help orientation. Each aliquot was covered with a glass coverslip after the addition of 10 mL of lugol's solution to kill the *Ancylostoma* spp. L_3. Then, they were counted and identified under a light microscope in magnification of 40x. The average of the *Ancylostoma* L_3 aliquots was calculated, which enabled the estimation of the average in the total suspension.

2.2. Scanning electron microscopy

The material preparation for the observation of larva/fungus interaction under the scanning electron microscopy followed the methodologies described by Nordbring-Hertz (1983) and Campos et al. (2008).

Seven 8 cm-diameter cellulose membrane discs (dialysis membrane) were used, containing proteins which weigh more than 12,000 Da, with filtering capacity of 640 mL/ft (Sigma-Aldrich®, USA). These discs were placed in a 100 mL Griffin cup that was filled with distilled water up to the 50 mL mark, closed with foil, and sealed with PCV film. The cups were later autoclaved at 121 °C for 15 min.

After being removed from the Griffin cup with tweezers, the discs were placed in seven 80 mm x 15 mm sterile Petri's dishes with 10 mL of 2% water-agar (2% WA Merse1, Brazil) so that the membrane covered the whole surface of the medium culture

and its border touched the glass wall of the Petri's dishes. On several sites of such a junction, small amounts of 2% WA were dropped with aid of a repeater pipette calibrated at 10 mL in order to seal it and prevent L_3 from escaping under the membrane.

Whole rice grains colonized by the nematode-trapping fungus *D. flagrans* (Isolate CG768) were stored at 4 °C in the Laboratório de Controle Biológico, Universidade Federal de Viçosa, Brazil, in the absence of light inside 5 mL vacutainer-like glass tubes filled up to their halves with blue silica-gel. A single rice grain colonized by this fungal isolate was transferred to the centre of 50 mm x 10 mm sterile Petri's dishes containing 5 mL of 2% WA. The plates were then incubated in the dark at 25 °C for 7 days. After the mycelial growth of this new culture, a 5 mm-diameter disc was replicated at the cellulose membrane surfaces of 80 mm x 15 mm Petri's dishes containing 10 mL of 2% WA medium (Nordbring-Hertz, 1983) that were afterwards incubated in the dark at 25 °C for 5 days. After that, the 5 mm-diameter culture disc was removed from the cellulose membrane surface and 20 mL of a suspension with nearly 10,000 *Ancylostoma* spp. L_3 were dropped on the *D. flagrans* culture. Two controls were performed, one with *Ancylostoma* spp. L_3 and the other with the nematode-trapping fungus *D. flagrans* (Isolate CG768).

During the first hours following the nematode inoculation, the Petri's

dishes colonized by the nematode-trapping fungus *D. flagrans* (Isolate CG768) were observed hourly under light microscope using magnification of 10x. After observing the capture of *Ancylostoma* spp. L_3 by this fungal isolate in a certain site of the membrane, marks were made on the outer surface of the Petri's dishes by using a fine-tip permanent ink maker, aiming to determine the *D. flagrans*/*Ancylostoma* spp. L_3 interaction time and find the initial capture site in further observations.

At the capture site, the cellulose membrane was cut into 7 mm^2 patches with aid of a scalpel blade. The patches had samples of recently captured L_3 (0 h) and samples collected 12, 24, 36 and 48 h after the beginning of predation. The patches were collected by using fine-tip tweezers and then fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer pH 7.4 at 4 °C, for 5 days, inside closed 1 mL cryopreservation tubes. Afterwards, the patches were removed and washed for three times in the same buffer and dehydrated through an ethanol series (30, 50, 60, 70, 95 and 100%) during 10 min for each concentration, and the highest one was repeated three times. The same material was submitted to Critical Point Drier (Balzers® CPD 020) by using carbon dioxide, covered with gold in Sputter-Coater (Balzers® SCA 010), after which electron micrographs were taken with a scanning electron microscope (LEO 1430 VP) at 20 kV.

3. Results

The initial formation of adhesive three-dimensional network traps, more robust than the vegetative hyphae, was observed 6 h after the *Ancylostoma* spp. L_3 were added to the Petri's dishes with cellulose membrane colonized by the fungus *D. flagrans* (Fig. 1 A and B). The three-dimensional networks, spaced at intervals along the length of the vegetative hyphae, were found in the whole cellulose membrane surface probably due to the fact that the L_3 were active and therefore dispersed on it (Fig. 1C). Initially, the traps did not show the network shape but were a simple loop of a lateral branch from a vegetative hypha that grew out, curved round and anastomosed with the parent hypha (Fig. 1A). New hyphal anastomoses of the branch from this primary loop or from another branch of the vegetative hypha were observed with the advance of the trap formation process, resulting in the adhesive three-dimensional network 12 h after the start of the interaction (Fig. 1B). It was not observed the formation of

traps in the control without *Ancylostoma* spp. L_3, but only the vegetative growth coating the surface of the membrane (Fig. 1D). The *Ancylostoma* spp. L_3 were active, moving with their characteristic serpentine motion, turgid and with integral membrane in the control without fungi (Fig. 1E).

Nearly 8 h after the addition of *Ancylostoma* spp. L_3 in the Petri's dishes, it was observed the capture of *Ancylostoma* spp. L_3 by the differentiated fungal hyphae, which had not evolved into a three-dimensional network yet (Fig. 1F). Pressure points could be seen on the cuticle of the *Ancylostoma* spp. L_3 recently captured by the trap hyphae, causing a small depression in the sheath where the penetration process began (Fig. 2G). In another situation, the *Ancylostoma* spp. L_3 captured was able to abandon the outer cuticle adhered in the fungal trap before being penetrated by the invasive hyphae (Fig. 2H). The majority of the larvae captured by the fungus *D. flagrans* were trapped at their cephalic portion (Fig. 2I); however, some *Ancylostoma* spp. L_3 were trapped at other parts of the body (Fig. 2J) and it was observed that some larvae were trapped even at both portions (Figs. 1F and 2G).

With the advance of the predation process, 12 h after the beginning of L_3 capture by the differentiated fungal hyphae, it was observed an increase in the size of the three-dimensional network and the enlargement of the infection bulb in the pressure points on the cuticle of the nematode (Fig. 2K and L). Randomly distributed rod-shaped bacteria were observed on the membrane surface and associated to fungus/larva interaction sites. No bacteria were observed at the interaction sites in the electron micrographs with recently captured larvae (Figs. 1F and 2G and I); however, high amounts of them were observed in electron micrographs at 12 h (Fig. 2J-L) and 24 h (Fig. 3M and N) after the beginning of capture.

In Petri's dishes with fungus/larva interactions, observed with a light microscope (40x and 100x), living *Ancylostoma* spp. L_3 were not visualized at 24 h after the beginning of predation. Besides, the larvae presented less turgor, which could indicate that they had possibly been penetrated by differentiated hyphae in the period between 12 and 24 h. That was also observed in scanning electron micrographs, with larvae showing less turgor, which may be probably a result of the dehydration caused by the translocation of the material from the nematode into the fungal infection bulb (Fig. 3M and N). It is important to emphasize that in the control Petri's dishes without fungus, the *Ancylostoma*

spp. L_3 were alive and vigorously moving during all evaluation period. Such observation was also performed under light microscopy with both 40x and 100x magnification.

Ancylostoma spp. L_3 were very dehydrated 36 h after the beginning of capture, exhibiting what could be the digestive hyphae inside the nematode coated by the cuticle (Fig. 3O). At that time, it was observed the exit of the nematode internal contents with the rupture of the cuticle (Fig. 3P). Forty-eight hours after the beginning of the capture, the nematodes were almost completely destroyed, and fungal invasive hyphae could be seen on the internal surface of the cuticle longitudinally split. Those were the remains of a previously whole nematode whose internal content was digested by the hyphae (Fig. 3Q and R). Two types of physical strength performed by the trap hyphae on the *Ancylostoma* spp. L_3 could be observed. The differentiated fungal hyphae were able to show constriction strength on recently captured L_3. It was possible to see the wrinkles caused by the smashing pressure on the cuticle (Figs. 2J and 4S), similar to the constriction caused by constricting ring traps, showing the speed and intensity of the initial capture processes to prevent the prey from escaping. Thirty-six hours after the capture, it could also be observed the emission of a hyphal branch from the network trap towards the L_3, tractioning, attracting and stretching it, since it was caught in the extremities by two other network traps (Fig. 4T).

Forty-eight hours after the beginning of the capture, the following reproductive and resistant structures of the fungus *D. flagrans* were observed: a young non-septate conidium, a mature septate conidium in a terminal conidiophore (Fig. 4U), and chlamydospores intercalated to the vegetative hyphae, with globular protuberances on their surface. In one of them, the beginning of a probable ‘‘germination’’ could be observed (Fig. 3V).

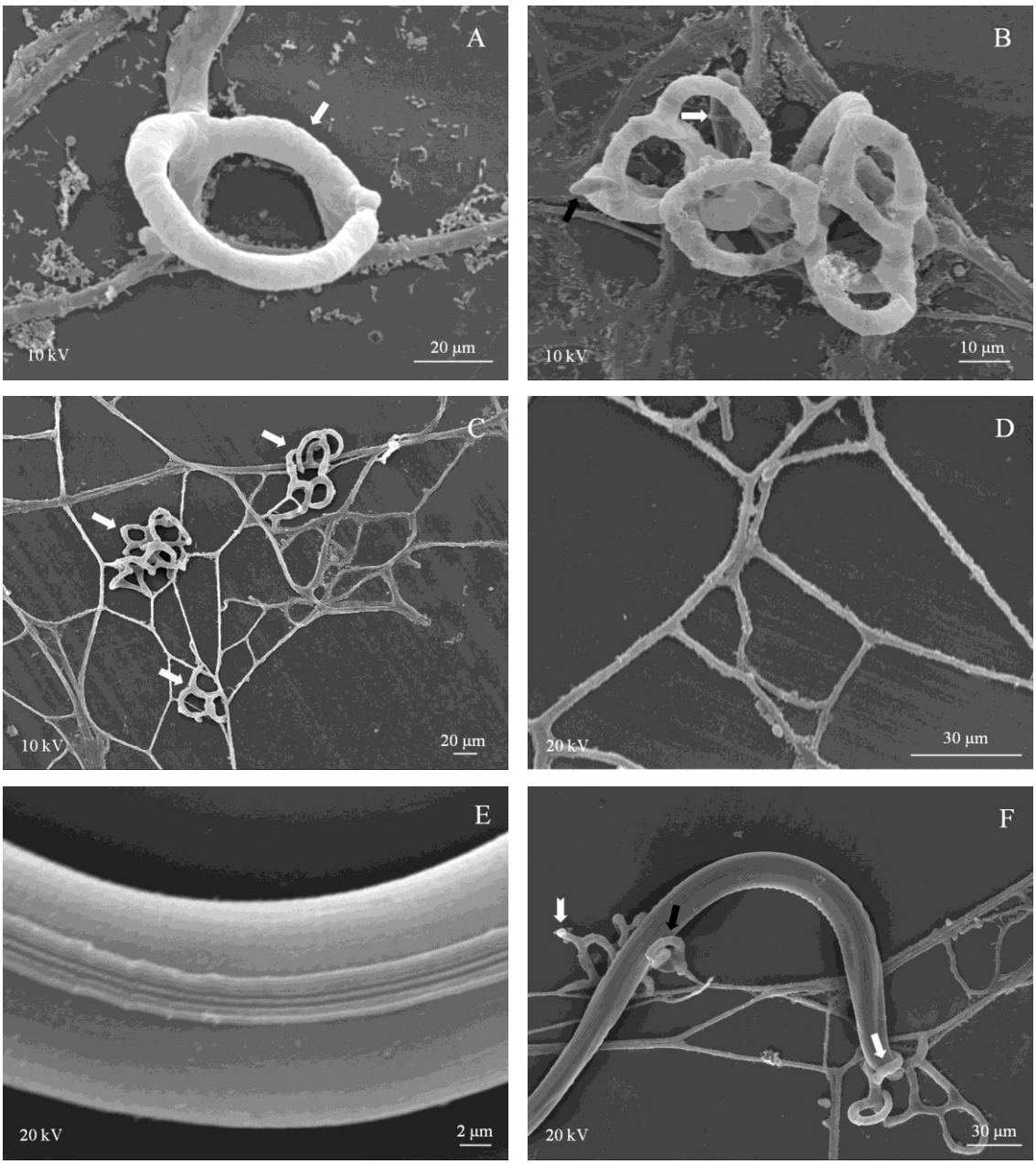


Fig. 1. Scanning electron micrographs of the trap formation process of the nematode-trapping fungus *Duddingtonia flagrans* (Isolate CG768) and the interaction against *Ancylostoma* spp. dog infective larvae (L_3) on the cellulose membrane surface. (A) Formation of the fungal trap (white arrow) after 6 h of the fungus/larva interaction; (B) fungal trap in anastomosis (white arrow), forming the scattered adhesive three-dimensional network trap and septate conidium of the *D. flagrans* fungus (black arrow); (C) scattered adhesive three-dimensional network traps (white arrow); (D) fungal hyphae of the control without *Ancylostoma* spp. L_3; (E) *Ancylostoma* spp. L_3 in the control without fungus; (F) *Ancylostoma* spp. L_3 captured, after 8 h of interaction with the fungus, at the cephalic portion (white arrow) and at the middle of the body (black arrow) by traps in the anastomosis process (dashed white arrow).

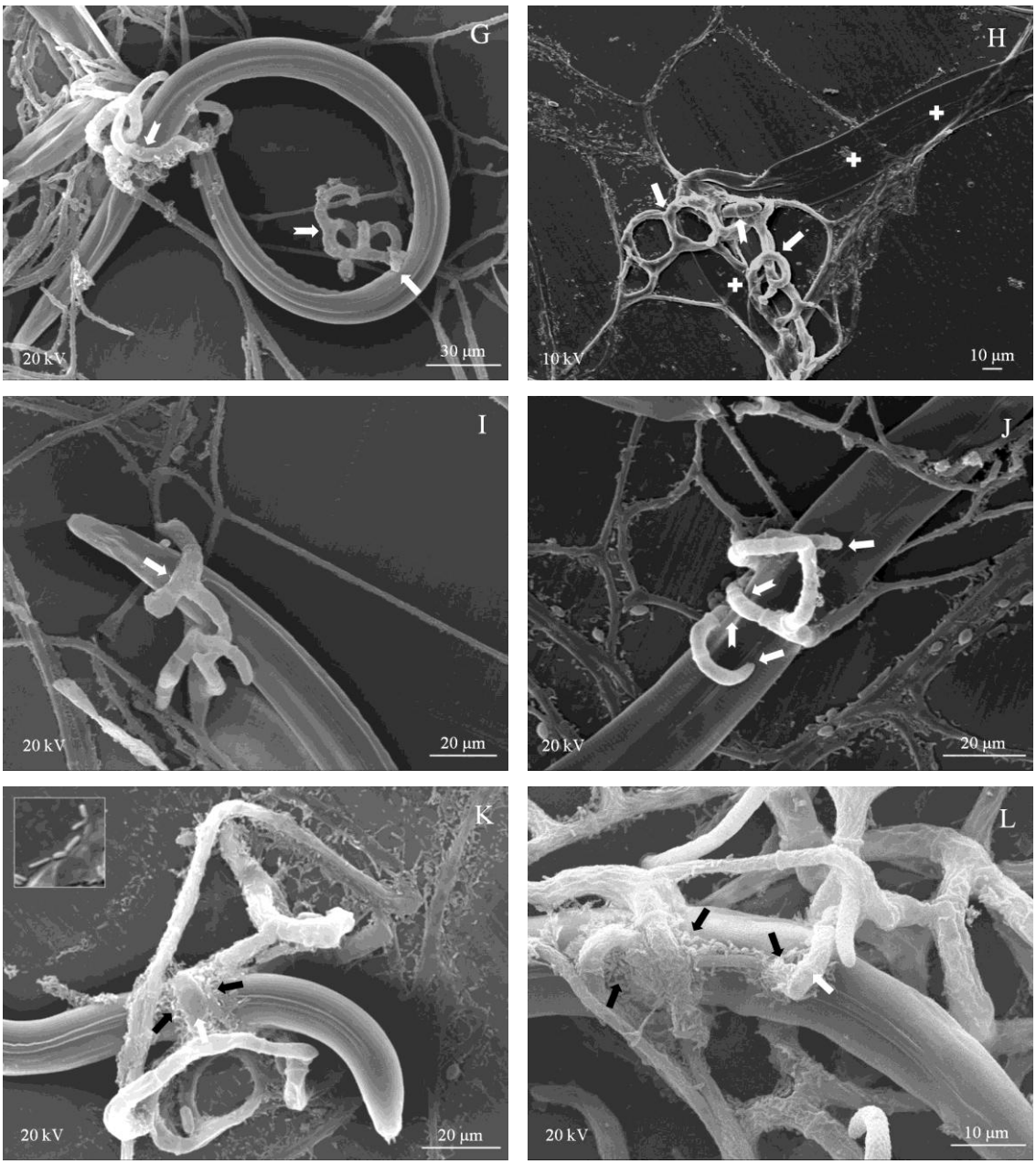


Fig. 2. Scanning electron micrographs of the interaction process between the nematode-trapping fungus *Duddingtonia flagrans* (Isolate CG768) against *Ancylostoma* spp. dog infective larvae (L_3) on the cellulose membrane surface. (G) *Ancylostoma* spp. L_3 captured after 8 h of interaction with the fungus by traps in the anastomosis process (dashed white arrow), pointing out the pressure on the cuticle caused by the modified hyphae (white arrows); (H) cuticle abandoned by *Ancylostoma* spp. L_3 (crosses), captured in an adhesive three-dimensional network trap (white arrows) and fungal conidium (dashed white arrow); (I) *Ancylostoma* spp. L_3 recently captured by the trap at the cephalic portion of the body after 8 h of interaction with the fungus, pointing out the pressure on the cuticle caused by the modified hyphae (white arrow); (J) *Ancylostoma* spp. L_3 recently captured by the trap at the middle of the body after 8 h of interaction with the fungus, pointing out the pressure on the cuticle (white arrows) and little constriction strength cuticle (dashed white arrows) caused by the modified hyphae; (K and L) *Ancylostoma* spp. L_3 12 h after the beginning of fungal predation. Note the swelling of the modified hyphae at the interaction sites (white arrow) and the presence of high amounts of Nematophagous Fungus Helper Bacteria (black arrows); rod-shaped bacteria detail (K – smaller figure).

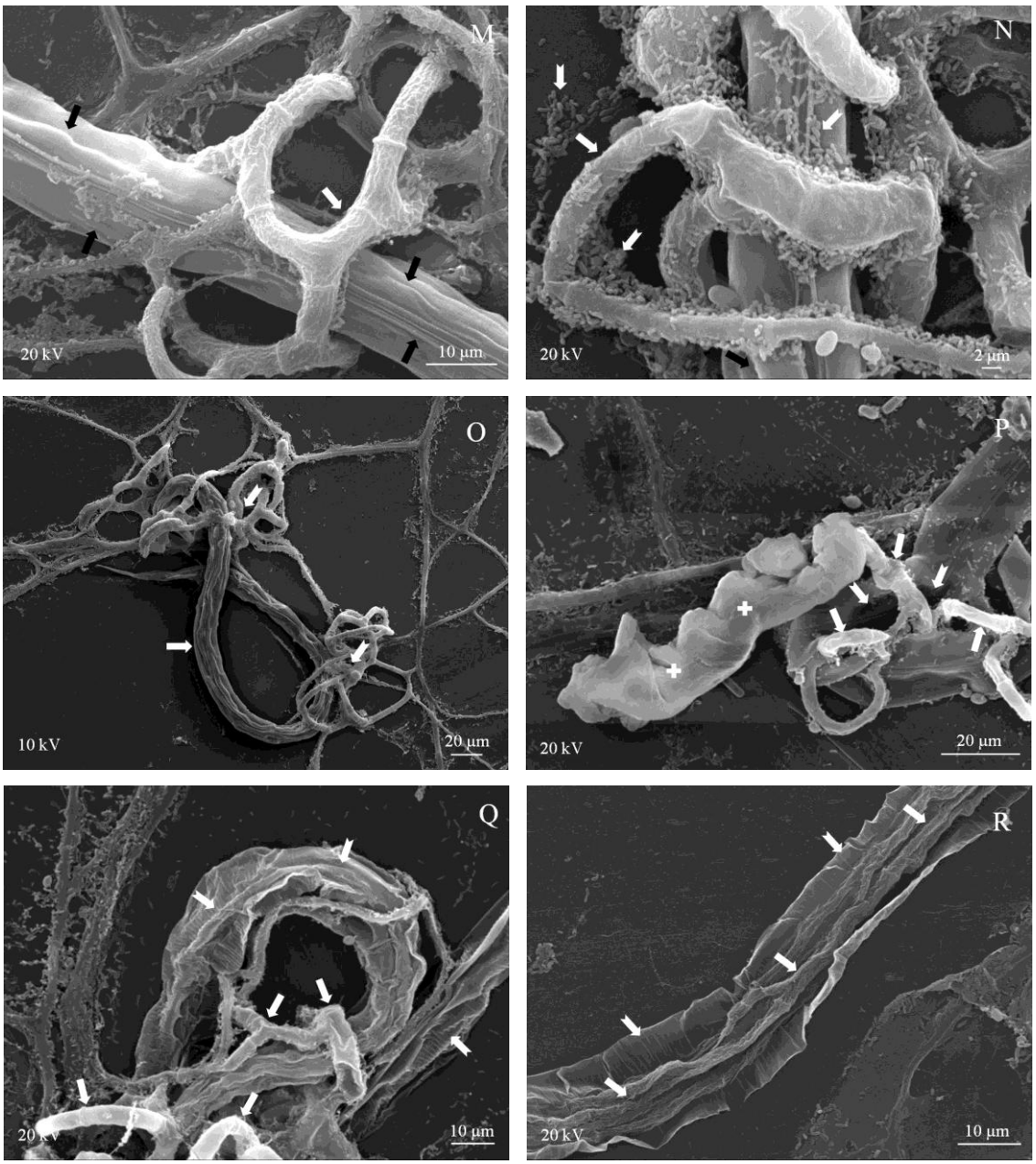


Fig. 3. Scanning electron micrographs of the interaction process between the nematode-trapping fungus *Duddingtonia flagrans* (Isolate CG768) against *Ancylostoma* spp. dog infective larvae (L_3) on the cellulose membrane surface. (M and N) *Ancylostoma* spp. L_3 24 h after the beginning of fungal predation by means of the adhesive three-dimensional network trap (white arrow) and the presence of high amounts of "Nematophagous Fungus Helper Bacteria" (dashed white arrow). Note the lower turgor of *Ancylostoma* spp. L_3 (black arrows); (O) *Ancylostoma* spp. L_3 36 h after the beginning of fungal predation by means of the adhesive three-dimensional network trap (dashed white arrow). *Ancylostoma* spp. L_3 is not turgid and the cuticle is wrinkled where suspected digestive hyphae are found inside *Ancylostoma* spp. L_3 (white arrow); (P) *Ancylostoma* spp. L_3 36 h after the beginning of predation by the fungus, showing the penetration of the nematode body by the modified fungal hyphae (white arrows), with cuticle breaking (dashed white arrow) and the exit of the nematode internal contents (crosses); (Q and R) *Ancylostoma* spp. L_3 at 48 h after the beginning predation by the fungus. Note the fungal hyphae (white arrows) that had their inner contents digested, leaving only the cuticle (white dashed arrows).

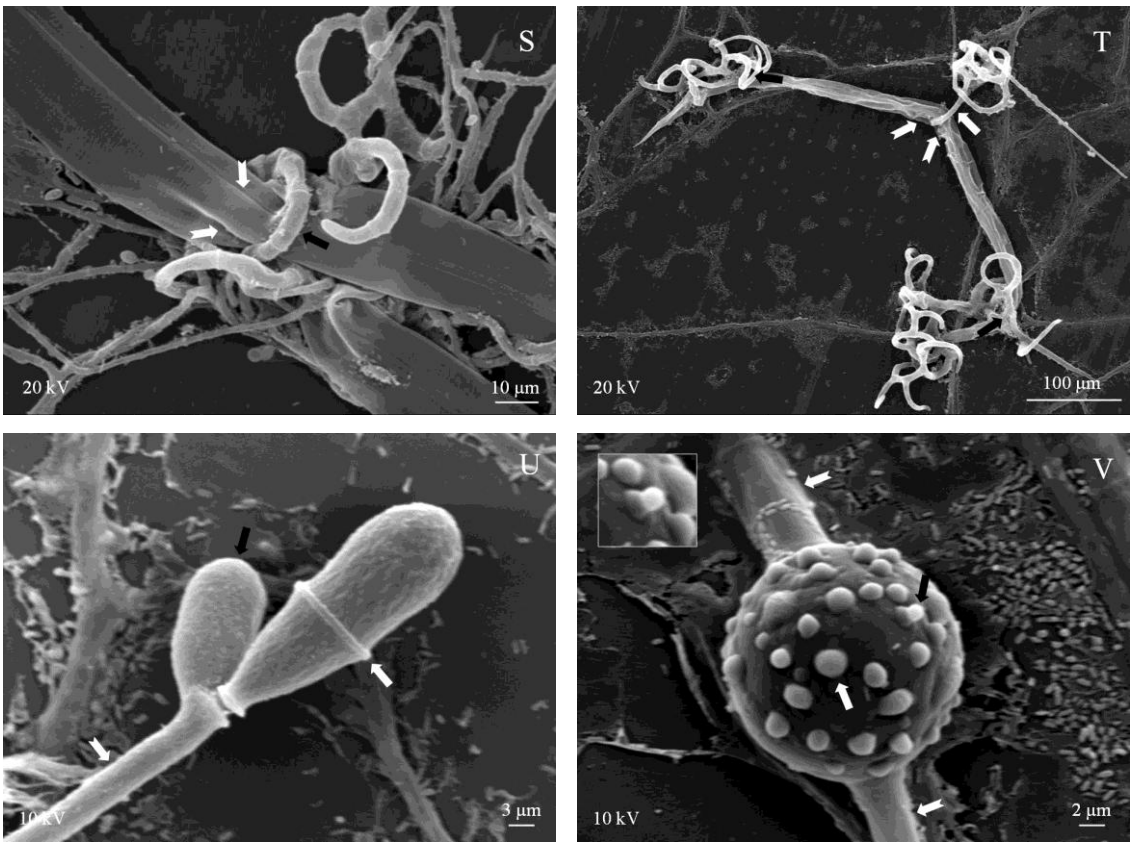


Fig. 4. Scanning electron micrographs of the interaction process between nematode-trapping fungus *Duddingtonia flagrans* (Isolate CG768) against *Ancylostoma* spp. dog infective larvae (L_3) on the cellulose membrane surface and of the reproductive and resistant structures of this fungus; (S) Constriction strength on the recently captured *Ancylostoma* spp. L_3 cuticle (dashed white arrows) showed by the modified fungal hypha (black arrow); (T) traction strength (white dashed arrows) showed by the modified hypha (white arrow) on the *Ancylostoma* spp. L_3 captured in the adhesive three-dimensional network trap (black arrows) after 36 h of predation; (U) elliptical shape conidia held on the conidiophore (white dashed arrows): non-septate young conidium (black arrows) and mature conidium showing a medium septum (white arrow); (V) chlamydospore intercalated to the vegetative hyphae (white dashed arrows), showing globular protuberances on the surface (white arrow), the beginning of a probable germination (black arrow), detail (smaller figure).

4. Discussion

The methodology used in the material preparation on cellulose membrane for analysis under scanning electron microscopy, proposed by Nordbring-Hertz (1983) and Campos et al. (2008), showed to be applicable and efficient for both material recording and the study of the interaction between the nematode-trapping fungus *D. flagrans* against *Ancylostoma* spp. L_3. The addition of 2% WA to the borders of the membrane prevented the *Ancylostoma* spp. L_3 from going under the cellulose membrane and maintained it on the surface, which favored their capture.

It is possible that the presence of *Ancylostoma* spp. L_3, or substances derived or excreted by them, stimulated the hyphal morphogenesis into traps by the fungus *D. flagrans* in Petri's dishes, with cellulose membrane on a 2% WA medium. This can be explained by the fact that a culture medium poor in nutrients, such as 2% WA, encourages nematophagous fungi to use nematodes as an alternative source of available nutrients (Scholler and Rubner, 1994), inducing the modification of the vegetative hyphae into nematode-trap structures (Nordbring-Hertz and Jansson, 1984; Nordbring-Hertz, 1988). Living nematodes are able to induce trap formation quicker than their extracts and their vigorous motility is probably involved in this process (Nordbring-Hertz, 1977; Jansson and Nordbring-Hertz, 1980).

According Scholler and Rubner (1994) the capture of nematode can be a symptom of nutritional deficiency in nematophagous fungi, since a culture medium with substrates containing adequate carbon and nitrogen concentrations, these fungi live as saprophytes forming few, or no trap structures. In this trial, carefully avoided agar-nutrient culture medium but only 2% WA, so the fungus could use only the *Ancylostoma* spp. L_3 as its nutritional source. However, according to Den Belder (1994), the formation of traps is a more complex response to the fungal needs. The enlargement observed in the differentiated fungal hyphae of the *D. flagrans* can be explained by the higher potassium level and higher turgor pressure inside this structure, according to Nordbring-Hertz (1977) and the resistance to high turgor is possible for the fact the trap wall is thicker than the vegetative hyphal wall (Nordbring-Hertz and Stalhammar-Carlemalm, 1978). These modifications allow the differentiated fungal hyphae to penetrate the nematode cuticle after capturing it, and grow within it, digest its inner contents and throw its own vegetative hyphae to the surface (Barron, 1977; Gray, 1987), as could be evidenced in the scanning electron micrographs recorded from 0 to 48 h.

In the present study, the time needed for the nematode-trapping fungus *D. flagrans* (Isolate CG768) to form traps was longer than that achieved by Nansen et al. (1986), who observed the presence of *Arthrobotrys oligospora* traps from 3 to 6 h after the addition free-living stages of *Cooperia oncophora* larvae on the surface of a colonized corn-meal-agar medium. However, it is important to remark that they use not only a different culture medium, but also a different fungus and nematode species. In a study conducted by Campos et al.

(2008), the trap formation by the fungus *D. flagrans* was observed 9 h after the initial interaction with *Haemonchus contortus* L_3. The same researcher reported that the first predated *H. contortus* L_3 by the fungus *D. flagrans* were observed 11 h after adding larvae to plates. Nevertheless, in present study, the first larvae in trap fungal structures were observed nearly 8 h after the addition of *Ancylostoma* spp. L_3 in the colonized Petri's dishes, which leads to the conclusion that the fungus *D. flagrans* was more skilled in trapping this nematode's larvae.

The expansion of the infective hyphae of the fungus *D. flagrans* observed in micrographs recorded at the first hours after capture may be possible the result of the translocation of material from the *Ancylostoma* spp. L_3 into the invasive structure or to an osmotic influx of water (Murray and Wharton, 1990). According to Den Belder et al. (1996) and Nordbring-Hertz et al. (2006), the fibrils of the cell wall of the traps become oriented towards the nematode, probably to facilitate the anchoring, after a penetration tube forms and pierces the nematode cuticle during the first hours of capture, being responsible for an intense traffic of cellular components at the point of contact with the nematode. In the micrographs was observed that the infective hyphae of *D. flagrans* in contact with the recently captured *Ancylostoma* spp. L_3 begin to swell, causing a small depression in the sheath, evidencing the physical pressure. Murray and Wharton (1990) reported that in these pressure points the complex penetration processes begin and this penetration force generated by the infective structure must be sufficient not only to penetrate the cuticle but also to overcome the high internal turgor pressure of the nematode itself. According to Nordbring-Hertz et al. (2006), not only the mechanical pressure but also the activity of hydrolytic enzymes, which solubilize the macromolecules of the nematode cuticle, could be also involved in the penetration processes. It is probably that the fungus *D. flagrans* produced enzymes that were involved in the penetration processes of the *Ancylostoma* spp. L_3.

According to Murray and Wharton (1990), the adhesive substances on the surface of the trap hyphae hold the nematode, nevertheless carbohydrate-binding protein (lectin) found on the fungal traps, which specifically bind to the carbohydrate receptor found on the nematode cuticle would probably be enough to hold the nematode (Zuckerman and Jansson, 1984; Jansson, 1987). It was observed a performance similar to constricting ring traps by the trap

structure of the fungus *D. flagrans* on recently captured L_3, revealing the intensity and speed of this fungus to prevent the prey from escaping and ensure its own nutrition. Nematodes may usually be attracted by compounds released from the mycelium and traps of nematode-trapping fungi (Nordbring-Hertz et al., 2006). However, in present study, it could be observed that the trap was able to emit a trap branch towards an *Ancylostoma* spp. L_3 that had already been captured, which may indicate that not only the fungus *D. flagrans* attracts these nematode larvae, but also these nematode larvae attracts this fungus. It was observed a higher number of *Ancylostoma* spp. L_3 captured at the cephalic portion, which may probably happen because this portion is the first part of the nematode body to get in contact with the traps when they are attracted by this trap structure. This observation can be explained by the fact that, sometimes, the carbohydrates on the surface are found scattered over the whole surface, but are frequently found at the head or at the tail, what gives us an explanation for the frequent binding of fungal traps to such nematode region, as reported by Jansson (1987).

Ancylostoma spp. L_3 stopped moving in less than 24 h in captivity, which leads to the conclusion that they were dead. According to Nordbring-Hertz (1988), this death is a consequence of its capture, because after adhering to the traps, the interaction process is irreversible, since the cuticle is penetrated by the fungus and the inner nutrients are digested. The fact that the *Ancylostoma* spp. L_3 presented less turgor in the scanning electron micrographs observed at 24, 36 and 48 h after the beginning of the capture is consistent with the translocation of water and other substances into the fungal infective hyphae, which would lead to a subsequent loss of the nematode movements, since they depend upon a high internal turgor pressure for locomotion (Wharton, 1986). In this study, the time of survival of the *Ancylostoma* spp. L_3, from the start of the capture, in which it showed motility in the traps, was longer than 12 h and shorter than 24 h. That may be explained by the difficulty of *D. flagrans* in penetrating the outer cuticle, a characteristic of the infective larvae of the animal parasitic nematode studied. The same does not happen to L_1 and L_2, but they quickly die (Gronvold et al., 1996a). Besides, according to Murray and Wharton (1990), the nematode and the fungus may have to be in a physical orientation favorable for the successful penetration of the cuticle by the invasive hypha, which may explain the length of the infection process in L_3 and the formation of

several infection bulbs. Nansen et al. (1986) observed that *C. oncophora* larvae showed less motility 20 h after being preyed by *Arthorbotrys oligospora*, but factors such as the fungus and nematode species used by them hampered comparisons with the current study.

Although the invasion in L_3 takes longer, in the present study, *Ancylostoma* spp. L_3 was penetrated and later destroyed, showing that the outer cuticle did not prevent the capture of this larvae by the fungus *D. flagrans*, which is in accordance with Murray and Wharton (1990), who recorded that the outer cuticle of the L_3 of parasite nematodes of domestic animals does not seem to protect them from predation by nematophagous fungi, since fungi are less skilled at preying ensheathed L_3. This was also verified by Araújo (1998), who observed that *Arthrobotrys* spp. isolates showed lower capacity to prey ensheathed *Cooperia punctata* L_3, revealing that there is a complex relationship between the surface receptors of free-living stages of the nematode and the ligands that are found on the cell wall of fungi.

The observation in the scanning electron micrographs of completely destroyed *Ancylostoma* spp. L_3, 48 h after the beginning of the capture, showed that the nematode larvae body was completely exploited by the hyphae and that this ability in consuming all internal content of the free-living stage of nematodes with the fungal biomass earnings and spore production can be a fungal strategy to increase their chances of environmental survival (Nordbring-Hertz, 1988). The conidia and chlamydospores that were observed in scanning electron micrographs are typical of *D. flagrans* species, which is in accordance with the morphology described by Van Oorschot (1985) and Gronvold et al. (1996b). As it was appreciated in the scanning electron micrograph observation, the predator fungus *D. flagrans* was able to destroy the infective *Ancylostoma* third stage larvae used in this experimental trial under *in vitro* conditions. These results make it possible to include *Ancylostoma* spp. of dogs in the range of the nematodes attacked by this predatory fungus. This ability of the fungus *D. flagrans* to capture, digest and therefore destroy free-living stages of several nematode species through adhesive three-dimensional network traps was reported by Larsen (2000).

The scattered rod-shaped bacteria observed on the cellulose membrane surface and at the fungus *D. flagrans*/*Ancylostoma* spp. L_3 interaction point were reported in others studies as helpers in the fungal activity. These bacteria

were called Nematophagous Fungus Helper Bacteria (NHB) and may be involved in predation, sporulation and pathogenicity systems of the nematophagous fungi, since that they produce substances that would act as molecular bridges between the fungi and nematodes larvae (Dupponois et al., 1998), which could explain the high amounts of such bacteria at the interaction sites after 12 and 24 h of *Ancylostoma* spp L_3 capture, observed in the present study. Due to such a fact, the study of the effect of these bacteria in association to the nematode-trapping fungus *D. flagrans* could provide a better understanding of their function in the fungus/larvae interaction process, since in literature only Campos et al. (2008) observed the association between these bacteria and this fungal species, although this researcher had worked with *H. contortus* L_3. The association of NHB with nematophagous fungi is also reported by Graminha et al. (2001), who studied the pathogenicity of the nematode-trapping fungi *Arthrobotrys musiformis* and *Arthrobotrys conoides* on *H. contortus* and *Ancylostoma* spp. L_3.

Further studies on the interaction between the *D. flagrans* isolates and *Ancylostoma* spp. L_3 for a better understanding of the physical, chemical and biological factors involved in this interaction could reveal important results, which would imply on the selection of more aggressive fungal isolates that could be used in biological control programs. The scanning electron microscopy proved to be an effective tool to understand of the ways nematophagous fungi act, revealing details of their trap structures and the interaction with nematode larvae. The use of transmission electron microscopy could provide complementary or additional information to the scanning electron microscopy about the nematode's penetration stage by nematophagous fungal hyphae, which would be crucial to understand these trap processes. The speed with which the nematode-trapping fungus *D. flagrans* (Isolate CG768) formed traps, preyed and destroyed the larvae shows the potential of this isolate as a biological agent for the control of dog *Ancylostoma* spp.

CAPÍTULO 3

Interaction between the nematode-trapping fungi *Arthrobotrys cladodes*, *A. robusta*, *A. oligospora* and *Duddingtonia flagrans* and *Ancylostoma* spp. dog infective larvae in soil microcosm

Abstract: The predatory ability of the nematode-trapping fungi *Arthrobotrys cladodes* (Isolate CG719), *A. robusta* (Isolate I31), *A. oligospora* (Isolate A183) and *Duddingtonia flagrans* (Isolate CG768) on *Ancylostoma* spp. dog infective larvae (L_3) was compared in soil microcosm at 25 °C at the end of 15 days, as well as the effect of different concentrations of *D. flagrans*, under the same conditions in two experiments. The inoculum concentration per grain of soil used in the fungal screening was 1,000 conidia. In the experiment I were used 500, 1,000, 1,500, 2,000 and 2,500 chlamydospores while in the experiment II were used 5,000, 10,000, 15,000, 20,000 and 25,000 chlamydospores. All tested fungi reduced the average of the *Ancylostoma* spp. L_3 recovered, compared to fungus-free control ($p < 0.05$). *A. oligospora*, *A. robusta* and *D. flagrans* were not different, but *D. flagrans* was selected because it produces numerous chlamydospores. The treatment concentrations were different from their respective fungus-free control (with 95% confidence intervals). The highest inoculum concentrations provided more consistent larval reduction than the lowest concentrations. The reductions in the larvae population in the experiment I were 40.47%, 44.09%, 44.74%, 47.44% and 47.89% and, in the experiment II, 58.28%, 68.80%, 70.31%, 70.79% and 72.18% from the lowest to the highest *D. flagrans* concentration, respectively. No difference on the reduction of the nematode was observed from 10,000 up to 25,000 chlamydospores. Probably, *D. flagrans* can play a significant role as a bio-control agent of dog *Ancylostoma* in the environment at 10,000 chlamydospores per gram of soil.
Keywords: predaceous fungi, *Ancylostoma* spp., dogs, biological control

1. Introduction

Gastrointestinal nematode parasites cause considerable costs of preventative or curative treatments in both domestic animals and human. Canines, mainly stray dogs, are frequently infected by species of genus *Ancylostoma*, a voracious blood-feeder nematode that causes subclinical infection or even death due to severe infections (Bowman et al., 2003). The soil contaminated with dog feces containing eggs of these parasites is also an important source of infection for humans, since the third-stage larvae, which are infective, can survive in the environment during several days (Soulsby, 1982). A high level of infection is observed among people (Macpherson, 2005),

particularly those at pediatric age (Hotez, 1989), who are infected through skin contact or ingestion of infective larvae from contaminated soil (McCarthy and Moore, 2000; Hotez et al., 2004).

Currently, in small animal practice, the preventative health measure and the classic treatment against *Ancylostoma* spp. have been performed exclusively with the use of anthelminthic drugs, which act only on adult hookworms inside the host (Lappin, 2001; Almeida and Ayres, 2006). However, a high level of anthelmintic resistance in *A. caninum* was reported (Jackson et al., 1987; Kopp et al., 2007), besides the possibility of environmental damage. Thus, alternative methods are required, such as biological control, which is characterized by the action of natural enemies (parasites, predators, pathogens, antagonists or competitors) that maintains the population of a target organism at a lower average than would occur in their absence, without causing alterations in the environment (Gronvold et al., 1996a; Larsen, 1999; Chen and Dickinson, 2004).

The biological component of the soil ecosystem limits or stabilizes the nematode population by mechanisms of competition, parasitism and production of toxic compounds (Stirling, 1991). Fungi play an important role in the function and dynamics of terrestrial ecosystems, directly influencing the structure of plant, animal, and bacterial communities through interactions that span the mutualism-parasitism continuum (Peay et al., 2008). Many animal-enteroparasitic nematodes develop their pre-parasitic phase in the soil. Epidemiologically, this is the most important phase, due to the possibility of re-infection or new infection occurrence (Soulsby, 1982). In this environment, they are particularly vulnerable to a wide range of soilborne fungi, called nematode-trapping fungi or predaceous fungi, found in all sorts of soil habitats, especially in organically rich environments (Larsen et al., 1994). They attack nematode larvae by means of trap structures and use them as an additional nutrient source (Gray, 1987; Nordbring-Hertz et al., 2006). Thus, nematode-trapping fungi may reduce the population density of nematodes in the soil and keep them under a threshold level (Gronvold et al., 1996a). Biological control of cattle, sheep and horses helminthiasis through nematode-trapping fungi was reported by many researchers (Waller and Faedo, 1993; Bird and Herd, 1995; Araújo et al., 2004). Recently, a high level of predation of *Ancylostoma* spp. L_3 was achieved by different nematode-trapping fungi (Maciel et al., 2006; 2009;

Carvalho et al., 2009).

Despite these reports, there is not enough information about whether nematode-trapping fungi can be used in strategies to control free-living forms of dog *Ancylostoma* in the environment. Therefore, this work presented results of the *in vitro* activity of the nematode-trapping fungi *Arthrobotrys cladodes* (Isolate CG719)*, A. robusta* (Isolate I31)*, A. oligospora* (Isolate A183) and *Duddingtonia flagrans* (Isolate CG768) on *Ancylostoma* spp. L_3 mortality in soil microcosm and investigates the effect of more infective fungi in low and high inoculum concentration, evaluating the possible use of them as biocontrollers of this dog-parasitic nematode in the environment.

2. Material and Methods

2.1. Fungal inoculum

Pure cultures of nematode-trapping fungi *Arthrobotrys cladodes* Drechsler (Isolate CG719)*, A. robusta* Duddington (Isolate I31)*, A. oligospora* Fresenius (Isolate A183) and *Duddingtonia flagrans* Cooke (Isolate CG768) were colonized, according the modified technique of Smith and Onions (1983), in integral rice grains and stored inside 5 mL BD Vacutainer® glass tubes (Becton Dickinson, Brazil) containing blue silica gel and kept at 4 °C in darkness. These tubes were closed with conventional stopper and sealed with polyvinyl chloride (PVC) transparent film. All fungi capture nematodes through three-dimensional adhesive networks. These tubes were closed with conventional stopper and sealed with polyvinyl chloride (PVC) transparent film. The identification of the species was based on the direct observation of the morphological characteristics, such as conidial size, morphology of conidiophores in micro-culture and trapping organs on nematode-infected culture, based on descriptions of Cooke and Godfrey (1964), Cooke (1969) and Van Oorschot (1985).

One integral rice grain, colonized by a single isolate, was transferred to the center of Petri dishes of 60 mm x 15 mm, containing 20 mL of 2% water-agar (2% WA) culture medium. Then, the fungi grew for seven days at 25 °C in the darkness, when mycelial discs of 5 mm diameter were removed from the fungal culture edges and transferred to Petri dishes of 90 mm x 15 mm containing V8-agar medium (200 mL of Campbell's V8 juice, 3 g of $CaCO_3$, 18 g

of agar and 800 mL of distilled water). Finally, the Petri dishes were kept at 25°C for ten days in the darkness to produce conidia. To induce *D. flagrans* chlamydospores, the Petri dishes were incubated under the same conditions, but for fifteen days (Fig. 1).

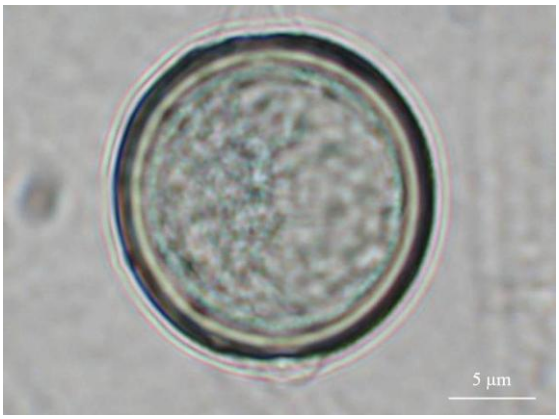


Fig. 1. Light micrograph of mature chlamydospore obtained from the micro-culture of *Duddingtonia flagrans* (Isolate CG768) show its thick-walled feature pattern.

Mycelial fragments and conidia were harvested by gently scraping the surface of the V8-agar with a brush after the addition of 10 mL distilled water. The conidial residue was then washed with 5 mL of distilled water taken from the medium surface. The fungal suspension was filtered through gauze (4 layers) and collected in a 50 mL Griffin glass, to reduce the mycelial fragments and obtain the conidial suspension. One drop of dispersant polysorbate (Tween® 80) was added in the fungal suspension under shaking for 2 minutes in a magnetic stirrer, in order to maximize the conidial separation. Shortly after, two aliquots of 10 µL were collected using a micropipette and placed on a Neubauer-counting chamber to estimate the conidia number per milliliter of suspension, according to Alfenas and Mafia (2007).

2.2. *Ancylostoma* spp. L_3

Ancylostoma spp. L_3 were obtained by incubating infected dog feces mixed with moist vermiculite kept at 26 °C for 10 days. Feces containing eggs were obtained from naturally infected stray dogs. These infected feces were identified by the method for the detection of hookworm eggs, according to Willis (1921). The infective larvae were extracted from the faecal cultures after using

the modified Baermann's technique (Baermann, 1917) for 12 hours and in this time were concentrated by gravity inside 5 mL BD Vacutainer® glass tubes (Becton Dickinson, Brazil) connected to the funnel. The sediment containing larvae was transferred to 15 mL BD Falcon® centrifuge polystyrene tubes (Becton Dickinson, Brazil), washed by centrifugation and resuspended in distilled water, five times at 1,000 rpm, for 5 minutes. The supernatant was gently aspirated by a plastic pipette and disposed at the end of each centrifugation.

The methodology described by Barçante et al. (2003) was used in order to filter the suspension, to eliminate debris and then obtain viable and active *Ancylostoma* spp. L_3 in a clean preparation. This selected suspension was vigorously homogenized and six aliquots of 10 µL were collected with a micropipette and placed on a slide marked with longitudinal lines to avoid the repeated counting of a same larva. Each aliquot was covered with a glass coverslip after the addition of 10 µL of lugol's solution to kill larvae. Thus, they were counted and identified under a light microscope with the magnification of 40x, allowing the estimation of the total number of larvae in the suspension. The motility of *Ancylostoma* spp. L_3 was checked by microscopical examination, before they were used in the experiment.

2.3. Experimental procedure

In order to evaluate the action of nematophagous fungi on *Ancylostoma* spp. L_3, in all controlled experiments, it was used a loamy sand soil (30% coarse sand, 17% fine sand, 14% silt and 39% clay) originated from the region of Viçosa, Minas Gerais, Brazil, presenting the following chemical characteristics: organic matter = 1.6 dag/kg; phosphorus = 12.6 mg/dm^3; potassium = 37 mg/dm^3; calcium = 4.1 cmolc/dm^3; magnesium = 0.7 cmolc/dm^3; aluminum = 0 cmolc/dm^3; pH = 6.4. This soil was sieved (5 mm mesh), placed inside polyethylene bags, and then pasteurized twice in water vapor for 1 hour, with an interval of 48 hours, in horizontal autoclave with vacuum valve open and water level controlled, before being used in experiments (Fig 2A).

An experiment was performed to compare the predatory activity of *Arthrobotrys cladodes* (Isolate CG719), *A. robusta* (Isolate I31), *A. oligospora* (Isolate A183) and *Duddingtonia flagrans* (Isolate CG768) on *Ancylostoma* spp.

L_3 in soil microcosm. Treatments consisted of the application of each fungal isolate and one fungus-free control. Based on the results of this experiment, two other experiments (I and II) were performed in order to evaluate the effect of five concentrations of *D. flagrans* chlamydospores on the nematode in study.

All experiments were assessed under the same conditions using 145 mL transparent polypropylene pots containing 40 g of soil with 25% of moisture (Fig. 2B). The soil was artificially infested with *Ancylostoma* spp. L_3 and conidia of the fungi (screening experiment) or chlamydospores of *D. flagrans* (experiments I and II). A standardized suspension of 100 µL, containing approximately 1,000 specimens of *Ancylostoma* spp. L_3, was placed in each pot. In the screening experiment, 1,000 conidia of each isolate per gram of soil were used. In experiment I, the chlamydospores ratios per gram of soil were 500:1, 1,000:1, 1,500:1, 2,000:1 and 2,500:1, while in the experiment II, the ratios were 5,000:1, 10,000:1, 15,000:1, 20,000:1 and 25,000:1. The chlamydospore concentrations required in each test resulted in different volumes of suspension. In order to compensate these differences, distilled water was added to the soil until reaching the standardized final volume of 500 µL in each one of the six replications per treatment. The fungus-free controls received the same volume of distilled water. Microorganisms were mixed with soil using a disposable wooden toothpick per replicate. Then, the pots were closed with a conventional lid and incubated for 15 days at 25 °C in an acclimatized darkened room. Every three days the pots were agitated to favor the interaction between microorganisms.

Fig. 2. (A) Soil pasteurization in autoclavate polyethylene bags; (B) Soil microcosm: 145 cm^3 transparent polypropylene pot containing 40 g of pasteurized soil.

At the end of the period, the larvae were recovered from the soil microcosm using the modified Baermann's technique, as described before. After discarding 3 mL of water without larvae from tubes, the *Ancylostoma* spp. L_3 were killed adding two drops of lugol's solution in the remaining volume containing the sediment. Then, this suspension of 2 mL containing dead larvae was transferred to Peter's counting slide and enumerated under light microscope (40x), according to Peters (1952).

2.4. Statistical method

The experiment was carried out once, arranged in a complete randomized design with six replicates per treatment. The larval recovery data were transformed into logarithm (log_{10}) in order to equalize variances and normalize the residuals prior to the analysis using ANOVA. For comparisons among isolates, the post hoc Tukey's multiple range test was performed to the level of significance of 5%. Statistical inferences were made on transformed data and back-transformed averages were presented. Descriptive analyses of panels were used to compare the effects between the chlamydospores rates and the reduction of nematode larvae by calculating averages with 95% confidence intervals. All statistical procedures were performed using the Statistica software, version 7.0 (Statsoft, 2004).

Larval recovery average values from the treatments were compared with the fungus-free control, and the trapping percentage was calculated as follows:

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

Where *RP* = reduction percentage; *x* = average of larvae recovered from the treatment; *y* = average of larvae recovered from the fungus-free control

3. Results

The fungal inoculum was able to germinate in pasteurized soil and trapping larvae. Results summarized in the Table 1 show averages and reduction percentages of *Ancylostoma* spp. L_3 recovered from the soil caused by the nematophagous fungi tested, after fifteen days of incubation, in

comparison to the fungus-free control. The medium value of the larvae recovered from the treated groups was lower than those from the fungus-free control ($p < 0.05$), at 1,000 conidia per gram of soil. The reduction percentages of *Ancylostoma* spp. L_3 recovered from the pasteurized soil, infested by fungi *A. cladodes*, *A. oligospora*, *A. robusta* and *D. flagrans* were 30.92%, 42.27%, 43.90% and 46.60%, respectively. The fungus *A. cladodes* presented low infectivity against *Ancylostoma* spp. L_3. There were no differences among the fungi *A. oligospora*, *A. robusta* and *D. flagrans*. However, since *D. flagrans* produces numerous chlamydospores (Figures 3 and 4), it was considered the more able fungus to be used in concentration tests.

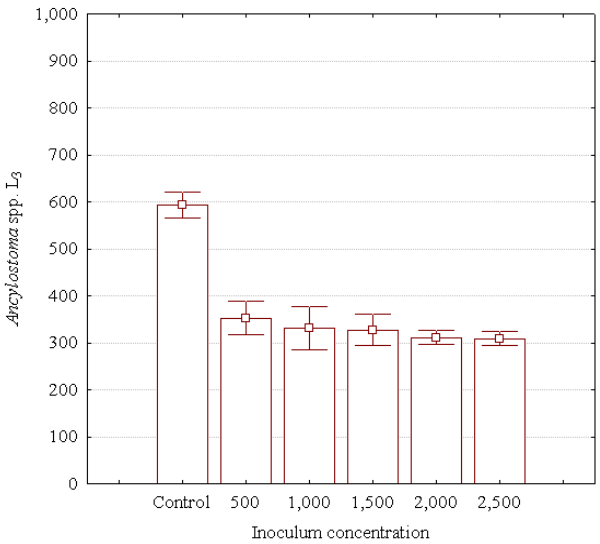
Table 1. Average values, standard deviation (±) and reduction percentage (%) of *Ancylostoma* spp. dog infective larvae (L_3) recovered from soil, initially with 1,000 L_3, by the Baermann method after fifteen days of interaction in microcosm containing nematophagous fungi *A. cladodes* (Isolate CG719), *A. robusta* (Isolate I31), *A. oligospora* (Isolate A183) and *D. flagrans* (Isolate CG768) in the concentration of 1,000 conidia/g of soil compared to fungus-free control

Fungal isolates	Average number	Reduction percentage (%)
A. oligospora	$352.67^{c} \pm 16.31$	42.27
A. cladodes	$422{,}00^{b} \pm 19.09$	30.92
D. flagrans	$326.17^{c} \pm 11.53$	46.60
A. robusta	$342.67^{c} \pm 17.40$	43.90
Fungus-free control	$610.85^{a} \pm 22.48$	-

Back-transformed averages followed by different superscripts in the same column are significantly different by the Tukey test ($p < 0.05$).
Reduction percentage = 100 - (treatment average x 100/control group average).

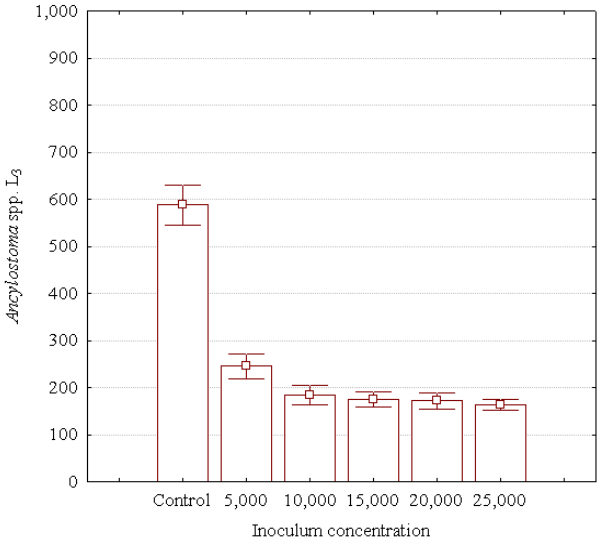
Figures 3 and 4 present the average values of *Ancylostoma* spp. L_3 recovered from the soil, containing different inoculum concentrations of *D. flagrans* chlamydospore. All chlamydospore rates were significantly different when individually compared with their respective fungus-free control (with 95% confidence interval). In general, the highest and the lowest predatory activity were achieved by adding low and high amounts of chlamydospores per gram of soil, respectively. Then, the high inoculum concentrations used in experiment II provided more consistent larval reduction than the low concentrations used in experiment I. Either way, irrespective of the concentration used, there was a

reduction in the number of *Ancylostoma* spp. L_3 recovered when compared to the fungus-free control. In experiment I, the results did not indicate differences among all *D. flagrans* treatments, probably due to the small increase in number of chlamydospores between each interval concentrations (Figure 3). This fungus caused a high and progressive reduction in *Ancylostoma* spp. L_3 harvested from the soil in experiment II. Nevertheless, there was no difference among concentrations from 10,000 up to 25,000 chlamydospores per gram of soil (Figure 4). It was observed a stronger reduction in the *Ancylostoma* spp. L_3 recovered in the concentration of 5,000 chlamydospores per gram of soil and the predation increased using 10,000 chlamydospores per gram of soil. However, the level of reduction in higher concentrations was similar to the 10,000 chlamydospores per gram of soil, tending to stabilize (Figure 4). This indicated that an increase in this concentration of *D. flagrans* chlamydospores in the soil microcosm did not caused more damage to the population of *Ancylostoma* spp. L_3. A higher inoculum concentration facilitated the distribution of the fungus, reaching a larger area in the soil microcosm and, undoubtedly, increased the chances of catching larvae, in comparison to the experiment I, with lower concentrations.



* Two independent averages are significantly different by the no-overlap between 95% IC.

Fig 3. Descriptive analysis by 95% confidence intervals (IC) of the average of uncaptured *Ancylostoma* spp. dog infective larvae (L_3) recovered from soil, initially with 1,000 L_3, by the Baermann method after 15 days of interaction in microcosm containing nematophagous fungus *Duddingtonia flagrans* (Isolate CG768) in five inoculum concentrations and respective fungus-free control of the experiment I.



* Two independent averages are significantly different by the no-overlap between 95% IC.

Fig. 4. Descriptive analysis by 95% confidence intervals (IC) of the average of uncaptured *Ancylostoma* spp. dog infective larvae (L_3) recovered from soil, initially with 1,000 L_3, by the Baermann method after 15 days of interaction in microcosm containing nematophagous fungus *Duddingtonia flagrans* (Isolate CG768) in five inoculum concentrations and respective fungus-free control of the experiment II.

There was a direct relationship between the effectiveness on the predation of *Ancylostoma* spp. L_3 and the inoculum concentration in the soil microcosm. In the experiment I, the reduction percentages of *Ancylostoma* spp. L_3 with increasing fungal inoculum concentration, compared to the respective fungus-free control were 40.47%, 44.09%, 44.74%, 47.44% and 47.89% in the inoculum concentration of 500, 1,000, 1,500, 2,000 and 2,500 chlamydospores per gram of soil, respectively. In the experiment II, with chlamydospores concentrations ten times higher than in the experiment I, the reduction percentages of *Ancylostoma* spp. L_3 were 58.28%, 68.80%, 70.31%, 70.79% and 72.18%, in the inoculum concentrations of 5,000, 10,000, 15,000, 20,000 and 25,000 chlamydospores per gram of soil, respectively.

4. Discussion

The current experiment showed that the incorporation of nematophagous fungi to the soil microcosm reduced the *Ancylostoma* spp. L_3 population, suggesting that these nematode larvae may have been used as food source.

The capacity of the nematophagous fungi to capture nematodes is well known (Gray, 1988; Larsen et al., 1994). However, the efficiency of nematode-trapping fungi in nematode control may vary (Galper et al., 1995). Besides, experimental conditions vary and hamper comparisons related to the capture efficiency of fungi on other nematodes, as, for example, on *Ancylostoma* spp. Nematode-trapping fungi *A. cladodes, A. robusta, A. oligospora* and *D. flagrans* reduced the number of *Ancylostoma* spp. L_3 in pasteurized soil, evidencing that they can be used in the environmental control of pre-parasitic forms of this dog-enteroparasitic nematode. Despite the poor results in screening experiment, the small percentage reduction of the larvae recovered in relation to the fungus-free control could be attributed to the interaction between the incubation period and the low ratio of conidia per gram of soil used. It is evident that the fungal inoculum concentration affects the performance of nematode-trapping fungi on nematodes. So, in higher inoculum concentrations, these fungi might probably reduce the population density of *Ancylostoma* spp. L_3. Furthermore, it must be pointed out that the effectiveness may also be affected by the increase of the period of interaction, resulting in a low number of recovered larvae.

It is not surprising that a significant improved reduction has been observed in current experiment, with the use of high inoculum amount of the fungus *D. flagrans*. This fungus has been successfully used as a biological control agent of gastrointestinal nematodes of domestic animals, due to its predaceous efficiency (Faedo et al., 1998; Baudena, 2000; Sanyal, 2000; Terrill et al., 2004). Its potential is due to the great production of thick-walled chlamydospores (Waghorn et al., 2003), which are highly resistant under adverse conditions (Larsen et al., 1991; Waller et al., 2001) and can originate hyphae and traps using only their considerable reserve of nutrients (Faedo et al., 2000; Skipp et al., 2002). According to earlier *in vitro* studies, *D. flagrans* entraps larvae through adhesive three-dimensional network traps, which are produced, in great amounts, in the presence of *Ancylostoma* spp. L_3 (Maciel et al., 2006; 2009). It should be emphasized that it attacks predominantly helminths that have short stage of development inside eggs, as the nematode here studied, whose larva of first stage is liberated in 20-24 hours, under appropriate conditions (Soulsby, 1982). Sometimes, the fungus survives for long periods, attacking also nematodes whose eggs hatch after 12-16 weeks, since, while the larva is in the egg, this fungus is not capable to capture it

(Faedo et al., 2000). The addition of large amounts of fungi to the soil is one of two general ways of applying the nematode biological control using nematophagous fungi (Nordbring-Hertz et al., 2006). The strong, intermediate and weak responses to chlamydospore concentrations were consistent in experiments with the fungus *D. flagrans*, with more evident predation of *Ancylostoma* spp. L_3 from 10,000 chlamydospores per gram of soil, with comparable efficiency in the other high concentrations. It is relevant that such virulence may be higher on L_1 and L_2 free-living forms, since Nansen et al. (1988) reported that these stages of development are more susceptible to action by nematophagous fungi.

The nematode mortality is influenced by the density of the traps, which are directly related to the number of conidia or chlamydospores, as pointed out by Gaspard and Mankau (1987). This was seemingly the cause of the damage level occurred on *Ancylostoma* spp. L_3 population in relation to its respective *D. flagrans* inoculum amount in soil microcosm. This is in agreement with Ribeiro (1999) and Maciel et al. (2009) who observed greater predation of nematodes with the increase of the fungal inoculum level, evidencing that the nematode control requests a great concentration of the antagonist. Since chlamydospores allow the establishment and survival of this fungus in the environment, it is the appropriate inoculum form to be used in biological control (Faedo et al., 2000), and that is why it was used in concentration tests with *D. flagrans*. According to Knox et al. (2002), *D. flagrans* could be detected in up to 30 cm deep below the soil profiles directly under the point of deposition, as the result of inadvertent chlamydospores relocation, by the activity of macroinvertebrates, such as arthropods and earthworms, or also by percolation into the soil with rain water. Thereby, once traps are stationary, it is obvious that the bigger the soil area containing chlamydospores of *D. flagrans* distributed homogeneously, the bigger the chances of interaction between the prey and its antagonists, regulating its population in this habitat. Based on this information, the movement of the fungal inoculum in artificial environment was guaranteed by the continuous agitation of the pots, and probably resulted in more interaction between the confronting microorganisms, ensuring the potential of a single predator on the prey available in the soil microcosm. Thus, due to the good fungus distribution on the soil profile, *Ancylostoma* spp. L_3 needed to migrate for a shorter distance towards network traps, whose form provides several contact

points for its prey. Nevertheless, the fungal predatory capacity is connected with both the capacity of the nematode to induce the hyphal morphogenesis in traps and the trapping efficiency of these structures (Nansen et al., 1988). This was observed in a previous *in vitro* survey using the same microorganisms here studied (Maciel et al., 2009). In the environment, the trapping ability of nematode-trapping fungi depends on the high inoculum level that will ensure the colonization of the soil through extensive mycelial growth, at intervals along which networks may be formed in high numbers (Gray, 1987; Gronvold, 1989). However, there is a delicate balance between hyphal development and trap formation and it is regulated by environmental factors (Scholler and Rubner, 1994), such as temperature, nematode density, oxygen tension, light and nutrient level (Gronvold, 1989). Above all, the presence of nematode larvae is the most important biotic factor involved in induction of trapping structures (Nordbring-Hertz et al., 2006). Moreover, the density of this target organism is also relevant to stimulate the predation by nematophagous fungi (Jaffee et al., 1992; 1993), such as *D. flagrans*, because when nematodes are in plentiful supply, perhaps between certain threshold densities, this fungus increase trapping rates (Morgan et al., 1997). In the present study, it seems that the number of *Ancylostoma* spp. L_3 used in soil microcosm was sufficient to stimulate the food-finding mechanism of fungus to capture them.

The pasteurization of the soil would eliminate the background fungi, but would create an overly artificial environment for the study of organisms with saprophytic potential. Differences in environmental conditions between laboratory and field may be relevant in the expression of the predation of nematophagous fungi, and the evaluation of its potential is influenced by the conditions of the experiment. In soils previously treated, as the one used in the current experiments, which present fewer competitors, fungi can feed on substrates other than nematodes, but in soil under natural conditions, several microorganisms are present and compete for nutrients, so predaceous fungi will use nematodes as food source (Hayes and Blackburn, 1966; Gray, 1987). However, unlike the previous report, in the present study, *D. flagrans* attacked *Ancylostoma* spp. L_3 in the absence of competitors with greater virulence. Such behavior indicated the importance of nematodes as suitable food, suggesting that this antagonist could be used against *Ancylostoma* spp. L_3 in the soil, irrespectively of the organic matter available.

Considering that nitrogen is a limiting factor for the growth and reproduction of nematode-trapping fungi, they get their nitrogen requirement from digesting the nematode's biomass (Barron, 1992; Barron, 2003). Therefore, it is clear that *Ancylostoma* spp. L_3 was the nitrogen source for *D. flagrans* in the soil microcosm. Thereby, it may mean that predatory fungi are not true saprotrophs, once two phases are necessary to supply them with the correct nutrients for growth – the saprotrophic phase and the predatory parasitic phase. This is in agreement with the findings of Cooke (1962) who indicated that the nematophagous habit supplement the saprophytic mode of life of fungi, but on the other hand, the nematode alone could not provide all the energy necessary for growth. Gray (1985) also reported that nematodes are necessary to initiate the formation of trapping organs, but fungi are incapable of remaining in a predaceously active state in the absence of an organic energy source other than nematode population. Adhesive network-forming fungi, as *D. flagrans*, may develop a saprophytically survival in the environment in the absence of prey, using the available organic matter for nutrition (Larsen et al., 1991). The relevant trapping activity of *D. flagrans* on *Ancylostoma* spp. L_3, associated with its noteworthy ecology, described above, suggests that this fungus would be able to maintain its predaceous efficiency in the hemiedaphic zone; rich in organic matter, moisture, soil nutrients and microorganisms where nematodes are available (Gray and Bailey, 1985). This is the appropriate habitat for the development of rhabditiform larvae (first and second stages), because they feed on bacteria and organic matter, and where the filariform larva (third stage) lives, encased in the cast cuticle of second molt, until depleting its reserves stored in intestinal cells (Soulsby, 1982) for up to forty nine days for the species *A. caninum*, if it does not find a host (Mark, 1975). Since nematophagous fungi are most likely to utilize nematodes only when their population is increasing in response to a temporary, most likely seasonal, increase in available soil nutrients (Gray, 1987), the purpose of using *D. flagrans* is to achieve prophylactic worm control, whereby future populations of pre-parasitic *Ancylostoma* spp. in environment are reduced. It is important to consider that this approach will almost certainly need to be applied in combination with other parasite control alternatives, including the timely and effective use of anthelminthics, because nematophagous fungi do not have any effect on worms established in hosts (Jackson and Miller, 2006).

The selection of possible nematode biocontrollers will have to take account the problems caused by antagonistic effects on the soil and also the target nematode (Gray, 1987). Despite the *in vitro* effectiveness, results showed that the *D. flagrans* is a potential antagonist of *Ancylostoma* spp. L_3, little is known about the biology and epidemiology of this fungus under natural conditions. This knowledge is necessary, because the competition among soil microbial communities in the environment is so intense that it would forbid the introduction of a new fungal species, mainly due to fungistasis (De Boer et al., 2003). So, the microbial activity is of paramount importance for the establishment of nematophagous fungi in soil because they will have to overcome such antagonism (Monfort et al., 2006). According to Gray (1983), the lack of success in the use of nematophagous fungi as biological control agents is generally due to the selection of the fungus, rarely based on its suitability to the habitat where the target nematode is found; but rather, on the ease of its isolation and subsequent culture or its relative ability in destroying nematodes under controlled conditions in the laboratory. The soil microcosm used in the present study can provide a model system for obtaining preliminary information about fungal viability and the extent of trapping on nematode species. Therefore, the promising fungi presented in this study should be the subject of new researches, either in controlled conditions or in natural systems. Furthermore, little is know about the environmental impact of the introduction of high fungal inoculum on non-target organisms in the soil ecosystem (Knox et al., 2002). While its contribution in the natural system is not fully understood yet, such statements indicate how future exploitation of *D. flagrans* for biological control should proceed, since it can be used strategically against *Ancylostoma* spp. without negative effects on the abundance of the mesofauna. Further studies on the ecological factors that affect the dynamic of the fungus *D. flagrans* in the environment may provide conclusive information to improve its efficiency in the nematode control and to determine its ecological impact, although no adverse effects were observed with the use of this fungus on non-target soil mesofauna organisms, as free-living nematodes, earthworms, microarthropods nor any other nematophagous fungi (Gronvold et al., 2000; Yeates et al., 2002).

The present work demonstrated a slightly better performance of the nematode-trapping fungus *D. flagrans* to reduce the number of *Ancylostoma*

spp. in pasteurized soil under laboratory conditions, but prospects for biological control depend on the methods to introduce fungal material in the environment, and on a sufficient amount to effectively reduce the parasite larval population. This fungus is an alternative as a biological control agent, but its concentration will determine the predation level. Future semi-natural and natural controlled experiments will demonstrate if *D. flagrans* can be used to fight off pre-parasitic forms of *Ancylostoma* spp. under natural soil conditions.

CAPÍTULO 4

Biological control of *Ancylostoma* spp. dog infective larvae in soil under semi-natural conditions

Abstract: Studies were undertaken to evaluate the predatory ability of the nematode-trapping fungus *Duddingtonia flagrans* on *Ancylostoma* spp. L_3 in soil pasteurized microcosm at 25 °C at the end of 15 days, as well as in natural soil at environmental temperature under greenhouse conditions by five assessments along of 30 days at intervals of 5 days among them. The fungus was inoculated by means of pre-colonized substrates at the standard rate of 10,000 chlamydospores per grain of soil. Milled maize and white rice were tested for massal production of *D. flagrans* chlamydospores, and the fungus produced 525,347 and 640,625 chlamydospores per gram of substrate, respectively, there was no difference between them. In microcosm assay, the fungus reduced the larval number recovered in comparison to the fungus-free control ($p < 0.05$). *Duddingtonia flagrans* alone, *D. flagrans* in white rice and *D. flagrans* in milled maize reduced larval population in 71.99, 78.38 and 79.41%, respectively. However there was no difference between *D. flagrans* substrates. The controls with fungus-free substrates, milled maize and white rice, caused small damage on the larval population, reducing it in just 8.28 and 13.39%, respectively, but no differences were observed among them and fungus-free control. In the biocontrol assay, the fungus was incorporated to the natural soil in pre-colonized milled maize. In all days of assessment, the average values of larvae recovered from fungus-free control were larger than those recovered from treatment with the fungus ($p < 0.05$). No differences were found between fungus-free substrate and fungus-free control when considering the average values of days 25 and 30 ($p < 0.05$). Considering the assessment days 10, 15, 20, 25 and 30 the reduction percentages observed in treatment with *D. flagrans* were 61.37, 73.23, 70.84, 64.50 and 56.99%, respectively, whereas in control with substrate the reduction percentages were 16.39, 27.99, 20.36, 14.45 and 9.20%. There was an inverse correlation between the larval recovered as a function of the days ($p < 0.05$). *Duddingtonia flagrans* showed predatory effect on plant nematodes present in natural soil. These results in semi-natural conditions demonstrate that *D. flagrans* can be considered as a non-chemotherapeutic alternative approach to the control of pre-parasitic forms of dog *Ancylostoma* spp.

Keywords: *Duddingtonia flagrans*, *Ancylostoma* spp., dogs, biological control

1. Introduction

Gastrointestinal parasitic nematodes, members of the family Ancylostomatidae, are blood-feeding intestinal parasites of mammalian hosts (Mulvenna et al. 2009) of considerable veterinary and medical importance (Datu et al., 2008). Some species of genus *Ancylostoma* are specific to humans or to animals, but others are zoonotic (Miranda et al., 2008a), such as *Ancylostoma caninum* and *A. braziliense* (Costa et al., 2008), since dogs are the major definitive host and humans, an accidental host (Davies et al., 1993). These ubiquitous hookworms generally live in the intestines of dogs and cats in tropical and subtropical regions, where conditions are conducive for hookworm survival (Traub et al., 2004; Zhan et al., 2008).

The potential role of companion animals as reservoirs for zoonotic diseases has been recognized as a significant public health problem worldwide (Schantz, 1994; Robertson et al., 2000). A large proportion of the *Ancylostoma* spp. population would be expected to be in refugia provided by free-living and somatic reservoir stages of the life cycle, as well as by parasites infecting free-ranging dogs and untreated pets (Kopp et al., 2007). These animals with patent infections put other canines at risk after contaminate the soil of public areas with feces containing eggs of the hookworm (Krämer et al., 2009). Contaminated soil is a major source of human infection, especially to the children (Macpherson, 2005).

Given the importance of the human-animal bond in modern society, and the high level of pet ownership, canine hookworms control is important to minimize its transmission to animals and human population (Schad, 1994; Bowman et al., 2003; Kopp et al., 2008a). While appropriate hygiene measures are essential, regular treatment of pets with an effective anthelmintic also has a key role in minimizing environmental contamination with these nematodes (Schad, 1994), but rapid reinfection and the high cost of treatment hamper control efforts (Albonico et al., 1995). Besides, resistance of the canine hookworm *A. caninum* to anthelmintic therapy with pyrantel is an emerging problem that has been reported (Jackson et al., 1987; Hopkins et al., 1989; Hopkins and Gyr, 1991; Kopp et al., 2007; Kopp et al., 2008b).

Therefore, there is an ongoing need for the development of safe, convenient and environmentally friendly products to reduce the potential for the

development of resistance (Colgravea et al., 2009). The aim of all prophylatic approaches to control is simply to limit host parasite contact to levels which do not compromise performance and/or welfare of animals by limiting the size of the parasitic population (Jackson and Miller, 2006). Alternative or complementary measures, when combined with other forms, of control may maintain effective control while reducing anti-helminthic usage comprise both the infective forms in the environment as the nematodes parasitizing the adult animal (Barger, 1999; Larsen, 1999; Stromberg and Averbeck, 1999), like biological control using nematophagous fungi by targeting larvae in environment (Sanyal and Mukhopadhyaya, 2003).

Considering the fact of nematophagous fungi are natural enemies of nematodes (Nordbring-Hertz et al., 2006) and commonly found worldwide, occurring in soil and environments rich in organic matter of several ecosystems (Gray, 1987), they can become part of an non-chemotherapeutic alternative approach (Larsen, 2000). Whereas that parasitic-nematode of animals spend part of their life cycle in soil (Soulsby, 1982), biocontrol agents have been investigated with aim to achieve a significant reduction in the larval population on environment, which will consequently result in a significant reduction of the worm burden in animals (Waller and Larsen, 1996; Wolstrup et al., 1996). Nematode-trapping fungi are able to trap and kill nematodes and the species *Duddingtonia flagrans* has been reported as the most promising to combat nematodes of several animals (Nansen et al., 1996; Fernández et al., 1997; Faedo et al., 2002; Chandrawathani et al., 2003; Dimander et al., 2003a). The potential of this natural nematode regulator is due to its capacity to produce abundant thick-walled restingspores, called chlamydospores, which have high degree of survival in harsh environmental conditions (anaerobic, enzymatic and thermal) such as animal gut (Larsen, 1999; Terril et al., 2004). These structures can originate hyphae and adhesive three-dimensional net-type traps using only its considerable reserve of nutrients (Faedo et al., 2000; Skipp et al., 2002).

Although a promising approach has been presented in feeding the grazing animals with *D. flagrans* chlamydospores (Larsen, 2000; Nordbring-hertz et al., 2006), the direct addition of these to the contaminated soil of backyards, public parks and playgrounds should be focused as the most appropriate way of applying biological control using nematophagous fungi as part of an integrated public veterinary and medical health system, in view that

the main canine hookworm reservoir are stray dogs (Heukelbach et al., 2002; Kopp et al., 2007). This fungal inoculum incorporation into the soil requires the addition of organic substrate (Cayrol and Ritter, 1984) because it is believed that the activity of nematophagous fungi might be stimulated by organic matter (Siddiqui and Mahmood, 1996). The biological control of canine nematodes has been neglected and there is little information about the effectiveness of nematophagous fungi on pre-parasitic forms of these parasites in the soil. Studies show that *D. flagrans* is antagonist of dog *Ancylostoma* causing significant reduction on infective larvae population (Maciel et al., 2006; 2009; Carvalho et al., 2009). This paper describes the results of experimentation carried out to enable assessment of the impact of *D. flagrans* on *Ancylostoma* spp. L_3 population in natural soil under greenhouse semi-natural conditions and provides information about two organic substrates favorable to inoculum production of this fungus.

2. Material and Methods

2.1. Fungal inoculum

Pure culture, previously isolated, of nematode-trapping fungus *Duddingtonia flagrans* Cooke (Isolate CG768), colonized in integral rice grains, was stored in the Laboratório de Controle Biológico de Nematóides, Universidade Federal de Viçosa, Brazil, at 4 °C in darkness inside 5 mL BD Vacutainer® glass tubes (Becton Dickinson, Brazil) containing blue silica gel, similar to the conservation technique of Smith and Onions (1983). These tubes were closed with conventional stopper and sealing with polyvinyl chloride (PVC) transparent film. The identification was based on the direct observation of the morphological characteristics, as the conidial size and the morphology of conidiophores in micro-culture, and trapping organs on nematode-infected culture, mainly following the descriptions furnished by Cooke (1969). *Duddingtonia flagrans* was carefully selected as candidate specie for introduction in soil by previous assay.

Mass production of *D. flagrans* chlamydospores was performed on cereals grains inside propylene bags with biological filter (Fig. 1). Inside of each bag were deposed 300 g of one single substrate, white rice grains or milled maize grains, and 40 mL of distilled water. Bags were sealed and sterilized for

30 minutes at 121 °C. Prior to fungal inoculation of the substrate, one integral rice grain colonized by *D. flagrans* was transferred to the center of Petri dishes of 60 mm x 15 mm, containing 20 mL of 2% water agar (2% WA) culture medium during seven days at 25 °C in the darkness. After mycelial growth, inside a vertical laminar flow cabinet, six medium disks of approximately 5 mm diameter, containing fungal mycelium, were removed from 2% WA fungal culture edges and transferred into the propylene bags containing substrate at environment temperature. Bags were again sealed to prevent contamination and *D. flagrans* grew on substrates as monocultures under acclimatized room at 25 °C in the darkness for 15 days. At intervals of 5 days until the end of the incubation period, blocks of colonized grains inside sealed bags were undone by pressure of fingers before shake this substrate with the purpose of ensure uniform fungal growth.

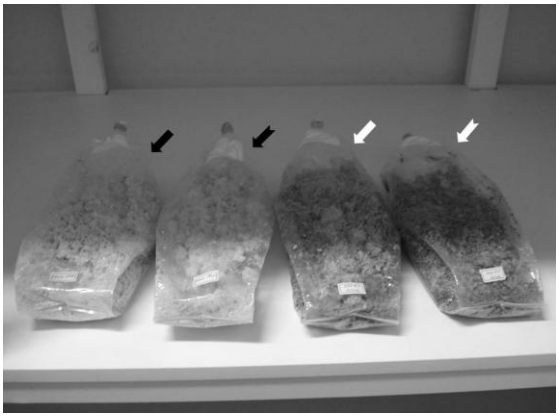


Fig. 1. Mass production of *D. flagrans* chlamydospores on milled maize and white rice inside propylene bags with biological filter. White rice (black arrow) and milled maize (dashed black arrow) with 10 days of fungal colonization. White rice (white arrow) and milled maize (dashed white arrow) with 15 days of fungal colonization, showing brown staining.

After the subsequent period of growth, the substrate was homogenized and 6 samples of 1 g were removed of each bag. Each sample was placed in an Erlenmeyer containing 10 mL of distilled water plus dispersant polysorbate (Tween® 80) to 0.2% (v/v) and was shaken for 2 minutes in a magnetic stirrer in order to maximize chlamydospores separation. The fungal suspension was filtered through gauze (4 layers) in order to reduce fragments, and collected in a 20 mL Griffin glass. Soon after, the number of chlamydospores per milliliter of the suspension, corresponding to the number of chlamydospores per grain of substrate, was estimate in aliquots of 10 µL placed on Neubauer-counting

chamber, according to Alfenas and Mafia (2007).

In order to obtain only *D. flagrans* chlamydospores, the colonized substrate was shaken with distilled water in vials closed with conventional lid. Then, fungal suspension was filtered through gauze (4 layers) and collected in a 500 mL Griffin glass to reduce the mycelial fragments. After 6 hours of the decanting by gravity, the chlamydospores of the bottom of the Griffin glass were harvested by the aspiration. The aspirated material was homogenized and the number of chlamydospores per milliliter was estimate as described before.

2.2. *Ancylostoma* spp. L_3

Ancylostoma spp. L_3 were obtained by incubating infected dog feces mixed with moist vermiculite kept at 26 °C for 10 days. Feces containing eggs were obtained from naturally infected urban street dogs. These infected feces were identified by the method for the detection of hookworm eggs, according to Willis (1921). Following incubation period, *Ancylostoma* spp. L_3 were extracted from the fecal cultures over 12 h using the modified Baermann technique (Baermann, 1917) through which they were concentrated by gravity inside 5 mL BD Vacutainer® glass tubes (Becton Dickinson, Brazil) connected to funnel. The sediment containing larvae was transferred to 15 mL BD Falcon® centrifuge polystyrene tubes (Becton Dickinson, Brazil), washed by centrifugation and resuspended in distilled water, five times at 1,000 rpm, for 5 minutes. The supernatant was gently aspirated by a plastic pipette and disposed at the end of each centrifugation.

The methodology described by Barçante et al. (2003) was used in order to filter the suspension, to eliminate debris and then obtain viable and active *Ancylostoma* spp. L_3 in a clean preparation. This selected suspension was vigorously homogenized and six aliquots of 10 µL were collected with a micropipette and placed on a slide marked with longitudinal lines to avoid the repeated counting of a same larva. Each aliquot was covered with a glass coverslip after the addition of 10 µL of lugol's solution to kill larvae. Thus, they were counted and identified under a light microscope with the magnification of 40x, allowing the estimation of the total number of larvae in the suspension. The motility of *Ancylostoma* spp. L_3 was checked by microscopical examination, before they were used in the experiment.

2.3. Experimental procedure

In order to evaluate the action of nematophagous fungus on *Ancylostoma* spp. L_3, in all controlled experiments, it was used a loamy sand soil (30% coarse sand, 17% fine sand, 14% silt and 39% clay) originated from the region of Viçosa, Minas Gerais, Brazil, presenting the following chemical characteristics: organic matter = 1.6 dag/kg; phosphorus = 12.6 mg/dm^3; potassium = 37 mg/dm^3; calcium = 4.1 cmolc/dm^3; magnesium = 0.7 cmolc/dm^3; aluminum = 0 cmolc/dm^3; pH = 6.4. The soil was collected from the soil profile (0-20 cm deep), sieved (5 mm mesh), and placed inside polyethylene bags. After that, soil was steam pasteurized twice in water vapor for 1 hour, with an interval of 48 hours, in horizontal autoclave with vacuum valve open and water level controlled, before being used in experiments. In the experiment under semi-natural conditions, the soil was air-dried at room temperature for one week to reduce its water content eliminating pre-parasitic forms of *Ancylostoma* spp., since they are susceptible to the dehydration (Soulsby, 1982).

2.3.1. Soil microcosm assay

The experiment was conducted to evaluate the predatory ability of nematode-trapping fungus *D. flagrans* on *Ancylostoma* spp. L_3 when the fungal inoculum was incorporated to the pasteurized soil without substrate or on pre-colonized substrates: white rice grains or milled maize grains. The experiment was performed in 145 cm^3 transparent polypropylene pot (Internal dimensions: height = 6 cm, mouth diameter = 6.7 cm, bottom diameter = 5.5 cm) containing 40 g of soil with 25% of moisture (25g water per 100 g of dry soil) (Fig. 2). A standardized suspension of 100 µL, containing approximately 1,000 specimens of *Ancylostoma* spp. L_3, was placed in each pot. Three treatments were established, one using only *D. flagrans* chlamydospores and the other two using individual application of a substrate pre-colonized by the nematode-trapping fungus. Three fungus-free controls were performed: two of them using the same substrates no colonized and other without any substrate. The uncolonized substrates were pre-autoclaved and the same amounts used in the treatments were used in the respective fungus-free controls.

Fig. 2. Soil microcosm: 145 cm^3 transparent polypropylene pot containing 40 g of pasteurized soil.

The nematode-trapping fungus produced 452,000 and 577,000 chlamydospores per grain of white rice and milled maize, respectively. Previous studies in soil microcosm show that no difference on the reduction of *Ancylostoma* spp. L_3 was observed using *D. flagrans* concentrations exceeding 10,000 chlamydospores per grain of soil. Thus, to result in approximately 10,000 chlamydospores per grain of soil, 0.69 g of milled maize or 0.88 g of white rice colonized by fungal were deposed in each pot for the designed treatment. Fungal inoculum, fungal inoculum substrates, substrates and nematodes were mixed to soil utilizing a disposable wooden toothpick per replicate. Pots were closed with conventional lid and incubated for 15 days at 25 °C in an acclimatized darkened room. Every three days, pots were agitated to favor the interaction between microorganisms.

At the end of the period, the larvae were recovered from the soil microcosm using the modified Baermann's technique, as described before. After discarding 3 mL of water without larvae, the *Ancylostoma* spp. L_3 were killed adding two drops of lugol's solution in the remaining volume containing the sediment. Then, this suspension of 2 mL containing dead larvae was transferred to Peter's counting slide and enumerated under light microscope (40x), according to Peters (1952).

2.3.2. Biocontrol in semi-natural conditions

In this subsequent experiment, a technique similar to previously used in soil microcosm assay was carried out to evaluate the predatory ability of

nematode-trapping fungus *D. flagrans* on target nematode in semi-natural conditions. The interaction between the target microorganisms was performed in 350 cm^3 transparent polypropylene pot (Internal dimensions: height = 8 cm, mouth diameter = 9 cm, bottom diameter = 7 cm) containing 200 g of untreated soil (Fig. 3). A standardized suspension of 250 µL, containing approximately 5,000 specimens of *Ancylostoma* spp. L_3, was placed in each pot. In treatment, the fungal inoculum was inoculated together with pre-colonize milled maize containing approximately 565,000 chlamydospores per grain. Thus 3.54 g of this pre-colonized substrate were added to result in 10,000 chlamydospores per grain of soil. Microorganisms were mixed to soil as described before. Two fungus-free controls were performed: one composed by the equal amount of the milled maize pre-autoclaved and no colonized and other without substrate. They were included to account for the probable activity of other antagonists of the target nematode, which would be naturally present in the raw soil. The pots were kept open, inside 500 cm^3 opaque plastic vase, under greenhouse conditions for a period of 30 days and protected from direct sunlight. The soil was moistened in alternate days, without flooding of water. These procedures were adopted based on the knowledge that the larvae of dog *Ancylostoma* live naturally in shaded and moist environments, because they do not resist to dehydration (Soulsby, 1982). Concurrently moisture is also necessary for the germination of *D. flagrans* chlamydospores (Knox et al., 2002).

Fig. 2. Soil microcosm: 350 cm^3 transparent polypropylene pot containing 200 g of untreated soil, inside 500 cm^3 opaque plastic vase, under greenhouse conditions

The assay was assessed on the tenth day and at intervals of five days, over thirty days period. In order to recovery of the larvae, the soil of each pot

was spread on one paper towel sheet (1 ply sheet, about 22 x 20 cm), and placed in a flat plastic sieve (20 cm in diameter). The sieve was adjusted on a plastic bottom plate filled with water at 41 ºC in contact with the surface of the liquid. This modified Baermann's apparatus (Baermann, 1917) was used to favoring the recovery of *Ancylostoma* spp. L_3 due to the increase of the contact area between soil and water. After 12 hours, the sediment containing larvae was poured into a funnel. The residue in the bottom of the plate was washed with jets of water from a plastic wash bottle on the top of the funnel. Then, the larvae were concentrated by gravity inside 5 mL BD Vacutainer™ glass tubes (Becton Dickinson, Brazil) connected to funnel for 6 hours. After discarding 3 mL of water without larvae from tubes, two drops of lugol's solution were added in the remaining volume containing the sediment to kill the *Ancylostoma* spp. L_3. Then, this volume of 2 mL containing dead *Ancylostoma* spp. L_3 was transferred for Peter's counting slide and enumerated as described before.

2.4. Statistical method

The experiment was carried out once, arranged in a complete randomized design with six replicates per treatment and fungus-free control. Descriptive analyses of panels were used to compare the production of chlamydospores between both substrates calculating averages with 95% confidence intervals. The larval recovery data were transformed into logarithm (log_{10}) in order to equalize variances and normalize the residuals prior to the analysis using ANOVA. For comparison of the predatory ability of nematode-trapping fungus between both substrates pre-colonized, the post hoc Tukey's multiple range test was performed to the level of significance of 5%. Statistical inferences were made on transformed data and back-transformed averages were presented. The regression analysis was applied to show the relationship between interaction days and reduction of parasite larvae. All statistical procedures were performed using the Statistica software, version 7.0 (Statsoft, 2004).

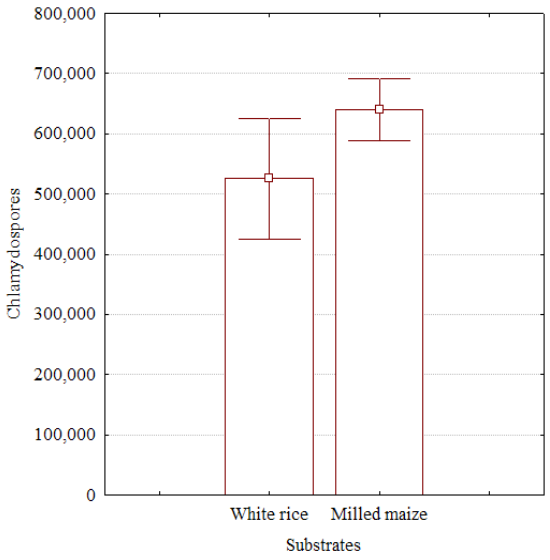
Larval recovery average values from the treatments were compared with the fungus-free control, and trapping efficacy (expressed as a percentage) was calculated as follows:

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

Where *RP* = reduction percentage; *x* = average of larvae recovered from the treatment; *y* = average of larvae recovered from the fungus-free control

3. Results

In the assessment inoculum production, milled maize and white rice were suitable substrates for the production of *D. flagrans* chlamydospores (Figure 1), with no difference between them (with 95% confidence interval). Average values of the chlamydospores count after 15 days of fungal growth were 525,347 and 640,625 chlamydospores per gram of milled maize and white rice, respectively. No contamination of the substrate by bacteria or fungi was observed in the sealed bags.



Two independent averages are significantly different by the no-overlap between 95% IC.

Fig. 1. Descriptive analysis by 95% confidence intervals (IC) of the average of chlamydospores of the nematode trapping-fungus *Duddingtonia flagrans* (Isolate CG768) produced in 1 grain of pre-autoclavated substrates after 15 days of growth inside 5 L propylene bags with biological filter under acclimatized room at 25 °C in the darkness.

Back transformed data on the predatory ability of *D. flagrans* based on the recovery of *Ancylostoma* spp. L_3, after 15 days of incubation, are showed in Table 1. Fungal treatment reduced the larval number in soil microcosm

compared with those in the fungus-free control ($p < 0.05$), with fewer larvae recovered. Treatments with *D. flagrans*, *D. flagrans* in white rice and *D. flagrans* in milled maize gave the greatest reductions in larval numbers, about 71.99, 78.38 and 79.41%, respectively. However, there was no difference between *D. flagrans* substrates ($p < 0.05$). Substrates control had a small impact on the larval population. The reduction percentages in larval numbers were 8.28 and 13.39% for fungus-free milled maize and white rice, respectively, but no differences were observed among them and fungus-free control

Table 1 - Average values, standard deviation (±) and reduction percentage (%) of *Ancylostoma* spp. dog infective larvae (L_3) recovered by the Baermann's method from pasteurized soil, initially infested with 1,000 L_3, treated with uncolonized substrates or *D. flagrans* (Isolate CG768) pre-colonized substrates in the concentration of 10,000 chlamydospores/g of soil, after 15 days in microcosm at 25 °C, in comparison to fungus-free control.

Treatments	Average number	Reduction percentage (%)
D. flagrans	$171.83^{c} \pm 5.64$	71.99
D. flagrans white rice	$132.67^{b} \pm 13.14$	78.38
D. flagrans milled maize	$126.33^{b} \pm 9.77$	79.41
Fungus-free white rice	$531.50^{a} \pm 57.96$	13.39
Fungus-free milled maize	$562.83^{a} \pm 63.41$	8.28
Fungus-free control	$613.67^{a} \pm 64.25$	-

Back-transformed averages followed by different superscripts in the same column are significantly different by the Tukey test ($p < 0.05$).
Reduction percentage = 100 - (treatment average x 100/control group average).

The Table 2 presents the average values of *Ancylostoma* spp. L_3 recovered from natural soil of the groups in over a period of 30 days in five assessments and the reduction percentage of larvae in relation to the fungus-free control. In all days of assessment, the average values of larvae recovered from fungus-free control were larger than those recovered from treatment with the fungus ($p < 0.05$). No differences were found between substrate control and fungus-free control, considering the averages of days 25 and 30 ($p < 0.05$). *Duddingtonia flagrans* demonstrated predatory activity from the first assessment on 10^{th} day from the start of the assay, due to the reduction of larvae recovered in comparison to the fungus-free control. This fungal activity increased on 15^{th} day and declined progressively from 20^{th} until the end of the experimental

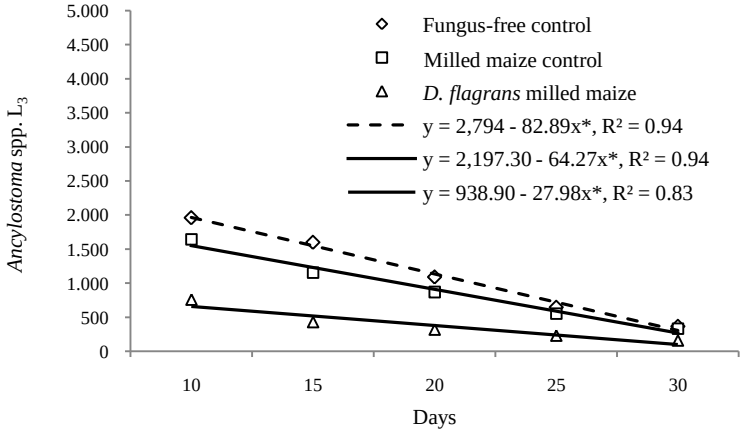
period, nevertheless resulted in low levels in the number of infective larvae. Comparable behavior throughout the observation period was demonstrated when the substrate was added to the soil, however with low reduction in number of recovered larvae. The single concentration rate (10,000 chlamydospores per grain of soil) showed a consistent reduction in larval population. In treatment with *D. flagrans*, the reduction percentages observed were 61.37, 73.23, 70.84, 64.50 and 56.99%, considering the assessment on days 10, 15, 20, 25 and 30, respectively. On the other hand, in substrate control the reduction percentages were 16.39, 27.99, 20.36, 14.45 and 9.20%, respectively from first to last assessment day established.

Table 2. Average values, standard deviation (±) and reduction percentage (%) of *Ancylostoma* spp. dog infective larvae (L_3) recovered by the Baermann's method from natural soil, initially infested with 5,000 L_3, treated with uncolonized substrate or *D. flagrans* (Isolate CG768) pre-colonized substrate in the concentration of 10,000 chlamydospores/g of soil, after 15 days at environmental temperature under greenhouse semi-natural conditions, in comparison to fungus-free control.

Treatments	Days				
	10	15	20	25	30
D. flagrans milled maize	759.00^{c} ± 82.09 (61.37)	429.17^{c} ± 69.46 (73.23)	319.00^{c} ± 70.56 (70.84)	230.50^{b} ± 24.73 (64.50)	158.83^{b} ± 23.54 (56.99)
Fungus-free milled maize	$1,642.67^{b}$ ± 162.91 (16.39)	$1,154.50^{b}$ ± 111.55 (27.99)	871.17^{b} ± 79.38 (20.36)	555.50^{a} ± 51.05 (14.45)	335.33^{a} ± 44.23 (9.20)
Fungus-free control	$1,964.67^{a}$ ± 215.10	$1,603.33^{a}$ ± 191.64	$1,094.00^{a}$ ± 98.42	649.33^{a} ± 53.83	369.33^{a} ± 41.92

Back-transformed averages followed by different superscripts in the same column are significantly different by the Tukey test ($p < 0.05$).
Inside the parentheses: Reduction percentage = 100 - (treatment average x 100/control group average).

The averages of *Ancylostoma* spp. L_3 recovered were used to estimate equations adjusted to the groups (Figure 4). All regression curves presented negative linear correlation coefficients as a function of the days with significant regression coefficient ($p < 0.05$) and high association degree ($R^2 > 0.83$). There was a high association degree between the average of larvae recovered and the fungal inoculum. The significant interaction between treatment and days showed that the activity of the fungus increased gradually, reducing the number of larvae recovered from soil over time. Seemingly, this reflects an increase in fungal effectiveness.



*Averages of the linear regression curve, statistically significant by the F-test ($p < 0.05$).

Fig. 4. Linear regression curves of the average of *Ancylostoma* spp. dog infective larvae (L_3) recovered from natural soil, initially infested with 5,000 L_3, treated with uncolonized substrate or *D. flagrans* (Isolate CG768) pre-colonized substrate in the concentration of 10,000 chlamydospores/g of soil and of the fungus-free control, by the Baermann's method after 10, 15, 20, 25 and 30 days at environmental temperature under greenhouse semi-natural conditions.

Four genera of plant nematodes were found in greatest numbers during assessment of the plots with substrate control and fungus-free control, and identified as *Helicotylenchus*, *Meloidogyne*, *Mesocriconema* and *Pratylenchus*. In fungal treated plots they also were observed, but in very small number indicating that is possible that the *D. flagrans* fungus trapping these plant nematodes. The air temperatures recorded over days are given in Fig. 5. During the experimental period the average temperature was 24.23 °C; the minimum temperature was 15.46 °C and the maximum temperature was 33 °C. It was observed the development of small plants in soil surface of the all pots. In the

first experimental days could be visualized in the fungus treated plots a hyphal development around of the substrate grains before of its decomposition in the soil.

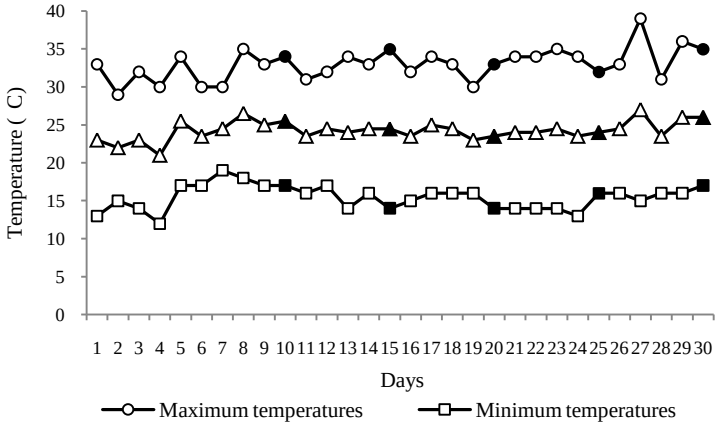


Fig. 5. Maximum, minimum and average air temperatures daily recorded during 1 month of experimental biocontrol of *Ancylostoma* spp. dog infective larvae (L_3) by nematode-trapping fungus *D. flagrans* (Isolate CG768) under semi-natural greenhouse conditions.

4. Discussion

The results showed the effectiveness of the nematode-trapping fungus *D. flagrans* on the control of *Ancylostoma* spp. L_3 in treated and untreated soils drastically reducing the initial population of this nematode. Conditions involved in soil microcosm don't represent those existing in the environment, where several ecological interactions are occurring, unlike of those in biocontrol assay using untreated soil at environmental temperature under greenhouse semi-natural conditions, getting closer to natural ecosystem. However, the soil microcosm using pasteurized soil represented a biological model and the initial phase of testing about the predatory ability of the nematode-trapping fungus on target nematode, in which biocontrol agents don't suffer interference from other soil organisms. Microcosm methodology was used because is suitable for assessing the interaction between microorganisms assisting in understanding processes, can manipulate population densities of nematodes and fungi, enables the use of appropriate forms of fungal inoculum in sufficient numbers of

which cannot be obtained for infesting large volumes of soil, besides achieve sufficient replication to obtain acceptable accuracy and precision of the results (Jaffee, 1996). In addition to being experimentally convenient, the use of small volumes of soil is biologically appropriate given the small size and limited motility of fungi and nematodes (Wiens, 1989). Meanwhile, the understanding natural soil processes involved in the biological control of pre-parasitic forms of *Ancylostoma* spp. by *D. flagrans* at the ecosystem level requires comparison of microcosm and field data.

Good results were observed when some environmental conditions were incorporated in the model of microcosm design. Biological control was evident in subsequent experiment under semi-natural conditions in response to the inoculation of *D. flagrans* with change in the pool of larvae reflected continuous decay rate of recovered larvae over days of assessement. Herein, undoubtedly, the antagonist maintained its activity until the end of the period capturing the infective larvae in the soil suggesting the survivability of the fungus. Knox et al. (2002) reported the *D. flagrans* persistence for 8-24 weeks after initial contamination in association with the soil under the point of fecal deposition, however this time can be prolonged until 20 months in dry environments (Gronvold et al., 1996a). Seemingly the uncolonized milled maize reduced the larvae population in soil. In addition to natural death of the larvae, this reduction might be explained by the stimulation of the microbial activity of microorganisms present in the untreated soil. The naturally occurring of nematophagous indigenous species considers the possibility that manipulation of the environment to enhance existing populations of fungi may also be feasible (Waghorn et al., 2002; Monfort et al., 2006). When considered in conjunction with the *D. flagrans* effect, such observation suggests that the population damage unlikely to be simply due to predation, but is possible that it has been the result of the combination of effects. These results are still somewhat surprising because suggest that even in the presence of organic matter the *D. flagrans* may be capable of trapping pre-parasitic forms of animal parasitic nematodes such as *Ancylostoma* spp. An interesting observation that should be taken in account was the caught of phytonematodes by *D. flagrans* showed the potential of this fungus as biocontrol agent these parasites that are pests in agriculture and horticulture, causing severe yield losses (Nordbring-Hertz et al., 2006).

The trap formation in nematophagous fungi is influenced by environmental factors such as nutrient level, pH, temperature, light and moisture (Nordbring-Hertz, 1973), but especially temperature (Bogus et al., 2005). In particular, the soil temperature is perhaps the main physical variable in terms of biological significance and should be taken into account when biological control systems are being developed (Stirling, 1991), because the optimum temperature for nematophagous fungi varies with each species influencing the percentage of arrested larvae (Morgan et al., 1997). It is likely that the air temperatures during the experimental period were relevant for the *D. flagrans* activity due to the low number of *Ancylostoma* spp. L_3 recovered from treated plots. According to Gronvold et al. (1996b) and Morgan et al. (1997), the optimal growth temperature for *D. flagrans* ranges between 25-33 °C. At temperatures lower than 25 °C it grows slowly (Larsen, 1991), presenting an upper limit of growth at 35 °C and not developing at 37 °C (Peloille, 1991). The highest temperature regimen in the present study exposed the *D. flagrans* to temperatures that exceeded 30 °C, but the maximum temperature in the soil was typically smaller than that recorded and may mean that the maximum air temperatures overestimate those real in the soil. Regarding the formation of tridimensional trapping nets, Gronvold et al. (1999) reported a higher trap formation rate at 30 °C than at 20 °C and observed that the slower development of *D. flagrans* at 10 °C does not decrease its trapping capacity, which remained high for a prolonged period of time, but can be compromised at temperatures persistently lower than 10 °C (Fernández et al., 1999). The trapping efficacy of *D. flagrans* may also be dependent on the duration of exposure to high temperatures rather than temperature alone (Kahn et al. 2007). In addition to the absolute effect of temperature, the effect of temperature fluctuation on trapping efficacy was demonstrated to be temperature dependent, there was reduction larvae between 15-25 °C (Fernández et al., 1999). The temperatures reported above are similar those recorded during experimental period between 14.5-33 °C. So, it is reasonable to state that temperature conditions and larval availability were conducive to optimal fungus activity which supports the results observed.

Mycelial growth and traps formation are processes that require energy prior to predation in non-spontaneous trap forming predators. According to Cooke (1969), the energy required for the mycelial growth and formation of

traps can be supplied by a source of carbohydrates. Thus, a factor that may be important for successful establishment of fungal biocontrol agents is the addition of a nutritional food base to the inoculum (Monfort et al., 2006). This procedure can increase the activity of fungal predators, because helps the germination of spores and mycelial growth, and favors the fungi in competition with other soil microorganisms (Payne and Lynch, 1988; Stirling, 1991), hence the *D. flagrans* was incorporated into the soil together with its substrate for growth. Thus, fungi with an energy source readily available probably would not have limitations to colonize the soil and form structures to capture nematodes (Dias and Ferraz, 1994; Cannayane and Rajendran, 2001). The soil can be more receptive to its indigenous isolates than to nonindigenous isolates. Apparently, the microbiota can determine the ability of nematophagous fungi to proliferate in soil (Monfort et al., 2006), probably due to fungistasis (Van Gundy, 1985) that can inhibit the germination, growth and action of the nematophagous fungus (Kerry, 1984). This antagonist effect on nematophagous fungi may due to microorganisms such as *Aspergillus terreus*, *Penicillium* sp., *Rhizopus* sp., *Trichoderma* sp. (Mankau, 1961; 1962), *Saccharomyces cerevisiae* (Rosenzweig and Ackroyd, 1984) and *Bacillus pimulus* (B'Chir and Namouch, 1988).

The fungistase occurs in untreated soil (Morgan-Jones and Rodriguez-Kabana, 1987), however the results showed that the possible presence of others microorganisms did not affect the *D. flagrans* efficacy, once that it presented consistent control. Therefore, a reasonable interpretation based on the above-mentioned results is that the milled maize was used by *D. flagrans* to establish in soil of pots resulting in the trapping of *Ancylostoma* spp. L_3 in this confined environmental system. It seems like that the growth of fungus tested was not inhibited in nonpasteurized soil and the reason for good results could be the ability of the *D. flagrans* to proliferate in soil. This is possible because predators fungi adhesive network formers are good saprophytes (Nordbring-Hertz, 1988) could be nurtured in the absence of other sources of nematode allowing their survival in soil (Gray, 1983). Furthermore, the resistant thick-walled *D. flagrans* chlamydospores, used as fungal inoculum in present study, have a long-term viability in soil under unfavourable environments (Larsen et al., 1991; Sanyal and Mukhopadhyaya, 2003) could germinate to form mycelium and trap in response to nematode activity (Skipp et al., 2002) providing high

trapping efficacies (Jackson and Miller, 2006).

Aspects such as the selection of an isolate with rapid growth, abundant sporulation and high pathogenic for the target organism, as well as the use of a substrate, minimally processed, quick and easy to use, low cost and viable on a large scale were taken into account in the present research based to the assumptions of Samsinakova et al. (1981) for the success in the large production and in the use of nematophagous fungi to the biocontrol. The methodology of fungal mass production employed using plastic bags with biological filter showed the practical feasibility to obtain large number of *D. flagrans* chlamydospores, providing a sterile environment for fungal growth without contamination of the substrates. Concurrently moistened white rice and milled maize grains were suitable substrates for the production of fungal inoculum in a short period of 15 days. They are related as good substrates for the production of *D. flagrans* chlamydospores (Waller et al., 2001), but other cereal grains as wheat, sorghum, and barley are also used for the inoculum production of this fungus (Sanyal, 2000; Sanyal and Mukhopadhyaya, 2003).

The Baermann's funnel is the method most used to extract nematodes from soil (Gray, 1987). The conventional Baermann's method was regarded practical and applicable to recovery the larvae added to the soil and determine the predatory efficiency of the nematode-trapping fungus. This is consistent with the fact that 61.37% of the total larvae added were recovered from soil microcosm when fungi were not added and this reflects the extraction efficiency in the absence of antagonists. It was observed that not all the larvae added to the soil were recovered, but this decrease was never associated with any signs of parasitism. The Baermann's apparatus modified also was efficient in recovery of larvae added to the untreated soil in biocontrol assay. The adaptation of this method, providing major contact area between soil and water, was relevant for the recovery of larvae from soil in the present study. However, the number of larvae recovered never exceeded 39% of total larvae added. Furthermore, the number of nematodes recovered from the nonpasteurized soil decreased in the subsequent days of assessment, recovering only 7% of them on the last experimental day. Understandably, since the soil was not sterilized, this larval reduction in control plots suggests the presence of indigenous antagonists of nematodes not used as experimental inoculum in addition to natural death of them over time.

The presence of *Ancylostoma* spp. L_3 in last day of evaluation showed the long survival this nematode. Up to a point, the soil may act as a reservoir of infective larvae (Krecek and Murrell, 1988) providing a more favourable environment for the development of larvae by protecting them from the more extreme environmental conditions on the soil surface (Bryan, 1976). Furthermore, for many parasitic nematodes, as *Ancylostoma* spp., be arrest at the L_3 facilitates survival in the environment (Datu et al., 2008). According to Hayes and Blackburn (1966) the predatory phase may continue until the nutritional requirement of the fungus is answered, and probably was the cause of the no extermination of the larvae in the microenvironment. Another explanation would be that the ability to attract the nematode by fungi could be influenced by spatial trap location in the soil since the uniform distribution would increase the chances of interaction between the microorganisms studied. However, the biological control may not lead to complete reduction of larval populations, it may reduce them sufficiently to prevent adverse effects, yet allow the stimulation of naturally acquired immunity (Barnes et al., 1995; Larsen et al., 1997). The effect of an artificial increase in nematophagous fungi in the environment is likely to be short-lived due to ecological factors that limit fungal populations in the field (Cooke and Satchuthananthavale, 1968). Despite this, there is the possibility that new incorporation of fungal inoculum may result in eliminating the larvae of the microcosm. The nematode trapping activity of *D. flagrans* is not specific to parasitic larvae and therefore there is the possible of perturbation of the soil nematode community (Dimander et al., 2003b). The introduction high levels this fungus in soil arouses the question as to adverse environmental impacts on soil indigenous populations in the ecosystem. However, studies with *D. flagrans* showed no effects on free-living nematodes, microarthropods and even on other nematode trapping-fungi (Yeates et al., 1997; Gronvold et al., 2000; Faedo et al., 2002; Knox et al., 2002). This indicates a future potential to use of *D. flagrans* as an organically acceptable alternative.

This study showed that biological control in nonpasteurized soil using *D. flagrans* presented satisfactory results in a small-scale of soil on *Ancylostoma* spp. L_3. The used concentration proved to be sufficient in controlling larvae under semi-natural conditions, but there is no guarantee that results obtained will always perform to the same degree when used in field situations provided

satisfactory control. Nevertheless, nematophagous fungi promising such as *D. flagrans* should continue being object of research, both under controlled conditions in the field, since it is necessary to know more of the ecological requirements, specificity, competitive capacity and establishment of introduced isolates. However, given that the fungi have no effect on established worm populations in host, this approach will almost certainly need to be applied in conjunction with other forms of control. An integrated management strategy, based upon epidemiological knowledge of the parasite involved, could be composed of two or more of the following elements: biological control agents, anthelminthics (minimal treatment) and hygienic measures. The results presented provide considerable encouragement for further research into the biological control of pre-parasitic forms of *Ancylostoma* spp. and suggest the need to develop some field trials determining biotic and abiotic factors that could interfere with the action of *D. flagrans* under adverse and varying climatic conditions through all seasons. Studies about formulation, delivery and inoculum concentration in natural soil also should be made. Significant potential exists for applying this fungus as biological control agent of *Ancylostoma* spp. L_3.

CONCLUSÕES GERAIS

Pode-se concluir que:

1. Os testes *in vitro* não representam as condições ambientais e por isso possuem limitações, mas são necessários no estudo da capacidade predatória de fungos nematófagos e na seleção de isolados mais infectivos, constituindo-se num método preliminar de estudo da interação do fungo antagonista com o nematóide alvo;
2. Existe variabilidade na infectividade sobre ancilostomatídeos pelas diferentes espécies fúngicas estudadas;
3. O isolado CG768 de *D. flagrans* tem elevada infectividade sobre larvas infectantes de ancilostomídeos, podendo destruí-las completamente em menos de 48 horas após a captura;
4. O isolado CG768 de *D. flagrans* é um promissor biocontrolador de ancilostomídeos de cães, pois reduziu o número de larvas infectantes destes nematóides recuperadas de solo não tratado em ensaio sob condições semi-naturais;
5. Grãos de arroz branco e de milho moído são substratos adequados para a produção massal de inóculo de fungos nematófagos como o isolado de *D. flagrans*, mas outros cereais devem ser testados;
6. A incorporação ao solo do fungo em seu substrato de crescimento é prático e pode viabilizar o estabelecimento do antagonista nematófago no ambiente por propiciar suporte nuticional inicial à sua fase pré-parasitária;
7. O isolado CG768 de *D. flagrans* tem potencial para reduzir a infestação ambiental com formas larvais pré-parasitárias de ancilostomídeos de cães, podendo ser uma alternativa viável e complementar ao uso de anti-helmínticos, ao controle populacional de cães de errantes e à educação da população;
8. Em vista dos percentuais de redução de L_3 de *Ancylostoma* spp. nos ensaios conduzidos, é necessário reforçar os resultados prosseguindo com estudos em solo sob condições naturais.

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