ANDRÉ RICARDO E SILVA

CONTROLE BIOLÓGICO DE NEMATÓIDES GASTRINTESTINAIS DE OVINOS, OVOS DE *Trichuris trichiura*, *Trichuris vulpis* E DE *Anoplocephala perfoliata* POR FUNGOS NEMATÓFAGOS

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 Scientia*e

VIÇOSA
MINAS GERAIS – BRASIL
2011

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T

S586c
2011

Silva, André Ricardo e, 1969-
Controle biológico de nematóides gastrintestinais de ovinos, ovos de *Trichuris trichiura*, *Trichuris vulpis* e de *Anoplocephala perfoliata* por fungos nematófagos / André Ricardo e Silva. – Viçosa, MG, 2011.
xvii, 82f. : il. ; 29cm.

Orientador: Jackson Victor de Araújo.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Parasitologia veterinária. 2. Saúde pública veterinária. 3. Helminto - Controle biológico. 4. Nematoda - Controle biológico. 5. Ovino - Doenças. 6. Animais domésticos - Doenças. 7. *Haemonchus contortus*. 8. *Trichuris trichiura*. 9. *Trichuris vulpis*. 10. *Anoplocephala perfoliata*. 11. Fungos nematófagos. 12. Homem - Doenças. 13. Cão - Doenças. 14. Equino - Doenças. 15. Biotecnologia. I. Universidade Federal de Viçosa. II. Título.

CDD 22. ed. 636.089696

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APROVADA: 25 de outubro de 2011.

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(Orientador)

À Deus pelo dom da vida, sem ele nada existiria!

Aos meus pais Luiz de Almeida e Silva e Geny da Silva “*in memorian*”.

À minha filha Carolina Vicentini de Azevedo Silva.

À minha família.

AGRADECIMENTOS

À Deus.

Aos meus pais e minha família.

A Universidade Federal de Viçosa e ao Departamento de Veterinária da UFV.

Aos órgãos de fomento FAPEMIG pelo financiamento do projeto e a CAPES pela bolsa de doutorado.

Ao Professor Jackson Victor de Araújo pela confiança, oportunidade, orientação e pelos ensinamentos indispensáveis a minha formação.

Ao professor Laércio dos Anjos Benjamim pela inestimável ajuda, dedicação e trabalho árduo no Núcleo de Microscopia Eletrônica e Microanálise (NMM) e no Departamento de Veterinária (DVT) /UFV.

Aos funcionários José Geraldo (Tuim) e Sr. Ademir pela amizade e contribuição valiosa em minha formação, nos experimentos a campo e no Laboratório de Parasitologia do DVT/UFV, enfim durante todos os momentos dessa trajetória.

A Lucinda, Aécio e Luiz Márcio pela contribuição no Laboratório de Patologia Clínica do DVT/UFV.

Ao Professor Dantas pela amizade e companheirismo, além dos ensinamentos e contribuição inequívoca a minha formação profissional.

Ao professor Joaquim Hérnan Patarroyo Salcedo pelas cobranças e pelos ensinamentos.

Aos professores Pacífico Antônio Diniz Belém, Ernane Paulino do Rego, Eduardo Paulino da Costa, Luís Augusto Nero e Abelardo da Silva Jr. pelos ensinamentos, pela amizade e orientações durante toda esta jornada, aos quais agradeço em nome de todos os professores da Universidade Federal de Viçosa que contribuíram direta e indiretamente por minha formação e por esta conquista.

Ao professor Adauto José Gonçalves de Araújo e a colega Daniela Leles de Souza da Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brasil, pelo fornecimento de material e pelas contribuições.

Aos colegas da pós-graduação, companheiros na busca incessante pelo conhecimento.

A todos os funcionários do DVT e do NMM da UFV, que contribuíram para a realização deste trabalho.

Aos Co-orientadores e membros da banca examinadora, pelas correções e sugestões na elaboração final deste trabalho.

A todos que direta ou indiretamente me ajudaram e motivaram na conquista desse objetivo.

Enfim agradeço a Deus por ter me dado esta oportunidade.

Meu,

Muito obrigado.

BIOGRAFIA

ANDRÉ RICARDO E SILVA, filho de Luiz de Almeida e Silva e Geny da Silva, *in memorian*, nasceu em 30 de novembro de 1969, em Viçosa, Minas Gerais. Em março de 1993, graduou-se em Medicina Veterinária pelo Departamento de Veterinária da Universidade Federal de Viçosa (UFV). Nessa data ingressou na indústria química e farmacêutica veterinária, Smithkline Beecham Laboratórios Ltda. Posteriormente, atuou na Pfizer Saúde Animal e em seguida na Indústria Química e Farmacêutica Schering-Plough S/A, exercendo atividades nas áreas técnicas e comercial dessas empresas. Após este período, atuou como autônomo, prestando consultorias a propriedades rurais de gado leiteiro nas regiões de Viçosa-MG e Colatina-ES, sendo que, nessa última, atuou como técnico do Projeto Educampo, em parceria com o SEBRAE daquele Estado. Realizou pesquisa junto à equipe do Prof. Dr. Sebastião Teixeira Gomes, do Departamento de Economia Rural, da UFV, na região central do estado do Tocantins-TO. O objetivo dessa pesquisa foi diagnosticar a situação da bacia leiteira da região central desse estado, para posteriormente possibilitar ações de revitalização e desenvolvimento dessa bacia leiteira. Após esta pesquisa foi convidado pela Nestlé, Dairy Partners Americas (DPA) para desenvolver e implementar o projeto Núcleo de Assistência Técnica Autorizada (NATA), na região do Centro-Oeste, mais especificamente, aos produtores fornecedores da indústria da região de Goiânia e Rialma no estado de Goiás. Em outubro de 2006, ingressou no Programa de Mestrado em Medicina Veterinária, no Departamento de Veterinária/UFV, submetendo-se à defesa de dissertação, em julho de 2008. Em agosto de 2008, ingressou no Programa de Doutorado em Medicina Veterinária, no Departamento de Veterinária/UFV, submetendo-se à defesa de tese, em outubro de 2011. Atualmente é revisor das revistas Veterinary Parasitology da WAAVP (World Association for the Advancement of Veterinary Parasitology e da Ciência Rural (UFSM). Durante o doutoramento participou como: Membro da Comissão Científica do III Simpósio de Pesquisa em Medicina Veterinária 2010 e III Semana da Pós-Graduação em Medicina Veterinária/UFV na área de Doenças e Biotecnologia. Coordenador Geral da Associação de Pós-Graduandos

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RESUMO

SILVA, André Ricardo, D. Sc., Universidade Federal de Viçosa, Outubro de 2011. **Controle biológico de nematóides gastrintestinais de ovinos, ovos de *Trichuris trichiura*, *Trichuris vulpis* e de *Anoplocephala perfoliata* por fungos nematófagos.** Orientador: Jackson Victor de Araújo. Co-orientadores: Laércio dos Anjos Benjamim e Abelardo da Silva Jr.

As helmintoses gastrointestinais provocam danos e podem matar ruminantes e outros animais, incluindo o homem. O uso indiscriminado de anti-helmínticos provocou a seleção de populações de helmintos resistentes aos diferentes grupos químicos utilizados. Fungos nematófagos, como *Duddingtonia flagrans, Monacrosporium thaumasium* e *Pochonia chlamydosporia* são utilizados no controle biológico das helmintoses gastrintestinais. No primeiro experimento, ovelhas foram tratadas com péletes contendo *D. flagrans* e *M. thaumasium* evidenciando que esses fungos podem ser usados como uma alternativa para o controle das nematodioses gastrintestinais de ovinos. No segundo experimento, *D. flagrans* (isolado AC001) e *M. thaumasium* (isolado NF34a) foram eficazes no controle *in vitro* de larvas de 3° estágio (L3) de *Haemonchus contortus* de ovinos e podem ser usados no controle biológico desse nematóide. No terceiro experimento, isolados de *P. chlamydosporia* (VC1 e VC4) tiveram papel importante na destruição de ovos de *Trichuris trichiura* de humanos. Além disso, em outro ensaio os fungos *D. flagrans* (AC001) e *M. thaumasium* (NF34a) predaram L3 de *H. contortus* de ovinos, mas nenhuma predação pelo fungo *P. chlamydosporia* foi observada. Os isolados de *P. chlamydosporia* (VC1 e VC4) podem ser controladores biológicos potenciais de *Trichuris trichiura* de humanos e os fungos *D. flagrans* (AC001) e *M. thaumasium* (NF34a) de *H. contortus* de ovinos. No quarto experimento, isolados de *P. chlamydosporia* (VC1 e VC4) demonstraram atividade ovicida ($p < 0,05$) em ovos de *T. vulpis* de cães. Os fungos *D. flagrans* (AC001) e *M. thaumasium* (NF34a) não mostraram efeito ovicida, mas *P. chlamydosporia* é um agente potencial para o controle biológico de ovos de *T. vulpis* de cães. No quinto experimento, o isolado do fungo nematófago *P. chlamydosporia* (VC1) destruiu ovos de *Anoplocephala*

perfoliata de equinos. Os fungos *D. flagrans* (AC001) e *M. thaumasium* (NF34a) foram efetivos no controle das nematodioses gastrintestinais de ovinos e os isolados VC1 e VC4 de *P. chlamydosporia* destruíram ovos de *T. trichiura* de humanos e *T. vulpis* de cães. O isolado VC1 destruiu ovos de *A. perfoliata* de equinos.

ABSTRACT

SILVA, André Ricardo, D. Sc., Universidade Federal de Viçosa, October, 2011. **Biologic control of gastrointestinal nematodes of ovine, eggs of *Trichuris trichiura*, *Trichuris vulpis* and *Anoplocephala perfoliata* by nematophagous fungi.** Adviser: Jackson Victor de Araújo. Co-Advisers: Laércio dos Anjos Benjamim and Abelardo da Silva Jr.

Gatrointetistinal helminthosis cause damage and can kill ruminants and other animals, including man. The indiscriminate use of anthelminthic caused the selection of populations of helminthes resistant to different chemical groups used. Nematophagous fungi are used in the biological control of gastrointestinal helminthosis, such as *Duddingtonia flagrans, Monacrosporium thaumasium* and *Pochonia chlamydosporia*. In the first experiment, sheep were treated with pellets with nematophagous fungi *D. flagrans* and *M. thaumasium* evidencing that the fungus can be used as an alternative for control of ovine gastrointestinal nematodiosis. In the second experiment, *D. flagrans* (AC001 isolate) and *M. thaumasium* (NF34a isolate) were efficient in controlling *in vitro* larvae in the third stage (L3) of *Haemonchus contortus* of ovine and can be used in the biological control of this nematode. In the third experiment, isolates of *P. chlamydosporia* (VC1 and VC4) had an important role in the destruction of eggs of human *Trichuris trichiura.* In addition, fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34a) preyed L3 of *H. contortus*, but no predation by *P. chlamydosporia* was found. Thus, isolates of *P. chlamydosporia* (VC1 and VC4) can be potential biological controllers of *Trichuris trichiura* in humans and *D. flagrans* (AC001) and *M. thaumasium* (NF34a) of *H. contortus* for ovine. In the fourth experiment, isolates of *P. chlamydosporia* (VC1 and VC4) showed ovicidal activity ($p < 0.05$) in eggs of *T. vulpis* of dogs. Fungi of *D. flagrans* (AC001) and *M. thaumasium* (NF34a) did not show ovicidal effect, but *P. chlamydosporia* is a potential agent for biological control of *T. vulpis* eggs in dogs. In the fifth experiment, isolate of nematophagous fungi *P. chlamydosporia* (VC1) destroyed eggs of *Anoplocephala perfoliata* in horses. *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were effective in controlling gastrointestinal nematodioisis of ovine and *P.*

chlamydosporia isolates (VC1 e VC4) destroyed eggs of *T. trichiura* in humans and *T. vulpis* of dogs. The isolate (VC1) destroyed eggs of *A. perfoliata* in horses.

(I) INTRODUÇÃO GERAL

Grandes perdas econômicas em pequenos ruminantes estão associadas ao parasitismo por helmintos gastrintestinais, principalmente *Haemonchus contortus*, que se alimenta de sangue. Prejuízos por essas infecções estão relacionados, principalmente, com a queda na produção, retardo no crescimento do animal, custos com tratamento médico veterinário e, em algumas situações, com a morte desses animais (ARAÚJO, 2006; SILVA et al., 2008; 2009).

No Brasil, grande parte da criação de ovinos é feita em regime de pasto ou parcial, levando a constante infecção por parasitos nas pastagens. As perdas econômicas mundiais anuais causadas pela infecção por nematóides gastrintestinais são estimadas em milhões de dólares, devido ao impacto que causam na produção de carne e leite e aos altos custos das medidas de controle, além de perdas na produção de lã e couro (ANUALPEC, 2003; SILVA et al., 2009).

O controle de verminoses em ovinos é geralmente feito com anti-helmínticos, os quais não têm sido eficazes no controle destes nematóides, devido à sua ação restrita aos parasitos adultos, ao aparecimento de resistência aos benzimidazóis, levamisois e avermectinas além da baixa possibilidade da formulação de novos compostos químicos de maior eficiência (ROCHA et al., 2006; SILVA et al., 2009).

Problemas com a resistência e ecotoxicidade enfatizam a necessidade de programas integrados de controle parasitário que assegurem saúde e segurança dos organismos vivos, por meio de tratamentos estratégicos baseados na epidemiologia, eliminação de vermifugações desnecessárias, utilização de pastoreio alternado e higienização de pastagens. Além disso, o uso continuado de uma mesma classe de anti-helmíntico deve ser evitado, como a rápida rotação de compostos e a utilização de doses inferiores às recomendadas (MOTA et al., 2003; THAMSBORG et al., 1999; SILVA et al., 2008; 2009; 2010a; 2011).

Pesquisadores buscam medidas alternativas para o controle das endoparasitoses de animais domésticos, visando à diminuição do emprego de quimioterápicos e, dos níveis de poluentes no ambiente e nos produtos

de origem animal. Medidas eficazes para o controle desses organismos devem ser buscadas, pois o controle químico tem problemas econômicos e ecológicos.

Controle biológico é uma alternativa viável e com resultados promissores *in vitro* e *in vivo* (LARSEN, 2000; SILVA et al; 2009; 2010a,b,c; 2011). Fungos nematófagos, constituem uma opção ao controle dos nematóides gastrintestinais de animais domésticos com ação concentrada no ambiente fecal para o combate de larvas de vida livre dos parasitos (GRONVOLD, et al., 1996; ARAÚJO et al., 1998; SILVA et., al 2009). O emprego destes fungos apresenta oportunidade de controle dos estágios de vida livre de nematóides nas pastagens, reduz as reinfecções e contribui para o controle das parasitoses (GRONVOLD et al., 1996; FAEDO et al., 1997; 2002; LARSEN, 2000; ROCHA et al., 2007; SILVA et., al 2009). O controle biológico das helmintoses gastrintestinais com fungos nematófagos visa diminuir a contaminação ambiental por estádios de vida livre dos parasitos que, em muitas situações, podem causar doenças tanto nos animais quanto nos humanos. Algumas helmintoses se destacam, pois parte de seu ciclo vital ocorre no ambiente e o homem pode ser hospedeiro acidental (SILVA et., al 2009; 2011).

A ação dos fungos nematófagos não é bem elucidada no controle biológico de ovos de *Trichuris* sp. e de *Anoplocephala perfoliata* (SILVA et., al 2010b,c; 2011).

Nematóides

Helmintos de interesse médico veterinário são divididos em dois filos: O Nemathelminthes engloba os Nematoda, e o Platyhelmintes é formado pelos Cestoda e Trematoda (URQUHART, et al., 1998).

A doença parasitária hemoncose é de grande importância para pequenos ruminantes, principalmente em regiões tropicais e subtropicais, sendo os ovinos e caprinos muito susceptíveis. Essa doença é causada pelo nematóide hematófago *Haemonchus contortus*, que gera lesões hemorrágicas no trato gastrointestinal (abomaso) e perdas na produção ou morte do animal, e, conseqüentemente, diminuição na lucratividade do produtor.

O ciclo biológico de *H. contortus* é direto, com uma fase pré-parasitária, tendo larvas (L_1 a L_3) no ambiente e uma fase parasitária (adultos machos e fêmeas) dentro do hospedeiro que se reproduzem e os ovos são eliminados juntamente com as fezes do hospedeiro. Os sintomas observados caracterizam-se, principalmente, pela anemia e hipoproteinemia, perda do apetite, emagrecimento, que pode resultar também na morte dos animais.

A parasitose cosmopolita trichurose causada pelo nematóide *Trichuris* sp., acomete o intestino grosso do trato gastrintestinal de animais domésticos, mamíferos silvestres e inclusive do homem. Possui um período de latência no solo antes de atingir o hospedeiro, não necessita de hospedeiro intermediário e apresenta ciclo biológico direto. Dípteros muscóides como *Chrysomya megacephala*, *Musca domestica* e outros, são vetores mecânicos de ovos de vários helmintos, inclusive *Trichuris* sp. presentes no corpo e no conteúdo intestinal das moscas (OLIVEIRA et al., 2002; SILVA et., al 2010b; 2011). Ovos de *Trichuris* sp. são eliminados com as fezes e dependendo da temperatura desenvolvem em um ou dois meses no ambiente para o estágio infectante L1 que quando ingerido pelos animais, torna-se adulto no intestino. Um dos aspectos mais importantes relacionados à epidemiologia deste parasito é que os ovos poderão sobreviver nas pastagens e no meio ambiente por três ou quatro anos. Os sinais clínicos dessa parasitose podem não ser aparentes, por isto seu diagnóstico deve ser feito com métodos laboratoriais como exame de fezes. O controle e o tratamento eficiente estão ligados respectivamente ao conhecimento de sua epidemiologia e à utilização de drogas anti-helmínticas (URQUHART et al., 1998). No entanto, os fungos nematófagos aparecem como uma possível ferramenta no controle desta parasitose (SILVA et al., 2010b, 2011).

Anoplocephala perfoliata é um cestóide, parasito do intestino grosso de equídeos. Os cestóides antes considerados parasitas não patogênicos, hoje são reconhecidos como potencialmente nocivos. Seus danos são relacionados com a localização dos mesmos que, em infecções maciças provocam nos equídeos, episódios de cólica e invaginação intestinal na junção íleo-cecal podendo levar o animal à morte. Ploglotes maduros são eliminados nas fezes e se desisntegram, liberando ovos que são ingeridos

por ácaros das forragens, nos quais se desenvolvem até o estágio de cisticercoide em 2-4 meses. Os cestóides adultos são encontrados no intestino dos equinos 1 ou 2 meses após a ingestão de foragens infectadas com ácaros (URQUHART et al., 1998).

Fungos nematófagos

Pesquisas com fungos que parasitam helmintos começaram com as observações de Lohde, em 1874, com o fungo endoparasita *Harposporium anguilullae* (KERRY, 1984). Fungos nematófagos como controladores biológicos vem sendo amplamente estudados no mundo para a redução de populações de nematóides no meio ambiente. Essas ações ocorrem através de organismos vivos que atuam como antagonistas naturais no ambiente (controle biológico). Fungos nematófagos são a alternativa mais promissora para o controle de helmintos. Eles são antagonistas naturais e capazes de promover a captura, a morte ou mesmo sua destruição, como endoparasitas, predadores, oportunistas (parasitas de ovos e cistos) e aqueles que produzem metabólitos tóxicos. A seleção de animais resistentes, vacinas e o controle biológico com fungos representam os últimos avanços contra as parasitoses gastrintestinais de animais domésticos (MANKAU, 1980; WALLER & LARSEN, 1993; SILVA et al., 2009; 2010a,b).

Os gêneros *Duddingtonia* e *Monacrosporium*, são os fungos predadores mais estudados (DIMANDER et al., 2003). *Duddingtonia flagrans* é a espécie mais promissora e estudada no controle das helmintoses de animais domésticos, com ação predatória por hifas adesivas simples e, além disso, possui dois tipos de estruturas (conídios e clamidósporos) intercalados em hifas maduras que permitem seu uso como controlador biológico, crescendo de forma lenta em temperaturas abaixo de 25° C, possuindo rápido crescimento em temperatura em torno de 30° C, (LARSEN, 1999).

As espécies do gênero *Monacrosporium* caracterizam-se por produzirem um único conídio por cada conidióforo, e com hifas septadas e ramificadas. *Monacrosporium thaumasium* age por redes adesivas, cresce de forma lenta em temperaturas abaixo de 25°C e crescimento ótimo em temperaturas de 28 a 30° C (BARRON, 1997).

O fungo ovicida *Pochonia chlamydosporia* é uma das espécies mais promissoras cuja ação é medida por: sem alteração; efeito tipo 1, efeito lítico sem prejuízo morfológico à casca do ovo, onde hifas são observadas aderidas à casca; tipo 2, efeito lítico com alteração morfológica da casca e embrião do ovo, sem penetração de hifas através da casca; e tipo 3, efeito lítico com alteração morfológica do embrião e da casca, além de penetração de hifas e colonização interna do ovo (LYSEK, 1976; 1978).

Duddingtonia flagrans e *Monacrosporium thaumasium* foram eficientes no controle de nematóides em laboratório e campo (MELO et al., 2003; TERRIL et al., 2004; SILVA et al., 2009;2010a). *Pochonia chlamydosporia* é pouco estudada, mas com atividade ovicida comprovada *in vitro* (ARAÚJO et al., 2008; BRAGA et al., 2007; 2008; SILVA et al., 2010c,d).

O controle biológico dos fungos nematófagos *D. flagrans* e *M. thaumasium* foi avaliado sobre os nematóides gastrintestinais de ovinos, essa última espécie (isolado NF34a) foi utilizada pela primeira vez a campo *in vivo* em ovinos. A primeira avaliação *in vitro* da ação dos fungos nematófagos *D. flagrans* (isolado AC001)*, M. thaumasium* (NF34a)) e *Pochonia chlamydosporia* (isolados VC1 e VC4) sobre ovos de *Trichuris vulpis* foi realizada. Esta é a primeira análise comparativa por microscopia eletrônica de varredura de fungos predadores *D. flagrans* (AC001) e *M. thaumasium* (NF34a) e ovicidas *P. chlamydosporia* (VC1 e VC4) sobre diferentes formas infectantes (L3) de *H. contortus* de ovinos e ovos de *T. trichiura* de humanos**.** Além disso, este é o primeiro relato da destruição de ovos de *Anoplocephala perfoliata* de equinos pela ação ovicida do *Pochonia chlamydosporia* (VC1).

OBJETIVOS DO PRESENTE TRABALHO:

1) Avaliar a efetividade dos fungos nematófagos *Duddingtonia flagrans* (AC001) e *Monacrosporium thaumasium* (NF34a) em formulação, pellets destes fungos em matriz de alginato de sódio, no controle biológico dos namatóides gastrintestinais de ovinos a campo.
2) Avaliar a atividade predatória dos fungos *Duddingtonia flagrans* (AC001) e *Monacrosporium thaumasium* (NF34a) sobre larvas infectantes (L3) de *Haemonchus contortus* de ovinos.
3) Demonstrar comparativamente por Microscopia Eletrônica de Varredura (MEV) a atividade *in vitro* dos fungos nematófagos ovicidas *Pochonia chlamydosporia* (isolados VC1 e VC4) e predadores *D. flagrans* (AC001) e *M. thaumasium* (NF34a) sobre ovos de *Trichuris trichiura* de humanos e larvas infectantes (L3) de *Haemonchus contortus* de ovinos.
4) Avaliar o efeito *in vitro* de quatro isolados de fungos nematófagos, *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34a) e *Pochonia chlamydosporia* (VC1 e VC4) sobre ovos de *Trichuris vulpis* de cães.
5) Avaliar o efeito ovicida do isolado VC1 de *P. chlamydosporia* sobre ovos de *Anoplocephala perfoliata* de equinos.

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(ii) Capítulo 1

BIOLOGICAL CONTROL OF SHEEP GASTROINTESTINAL NEMATODIASIS IN A TROPICAL REGION OF THE SOUTHEAST OF BRAZIL WITH THE NEMATODE PREDATORY FUNGI *Duddingtonia flagrans* AND *Monacrosporium thaumasium*

PARASITOLOGY RESEARCH, 105, Number 6, p.1707-1713, 2009.
DOI: 10.1007/S00436-009-1613-8

Abstract

Fungus can be used as an alternative for control of ovine gastrointestinal nematodiosis. Formulations in matrix of sodium alginate (pellets) of the nematode predatory fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* were evaluated in the biological control of sheep gastrointestinal nematodiasis. Three groups (1, 2 and 3), each one with eight sheep of the Santa Inês breed, at the ages of 15-48 months, were placed in paddocks of *Brachiaria decumbens* for five months. In group 1, each animal received 1g/10 kg of live weight (l.w.) of pellets of *D. flagrans* (0.2g of fungus/10Kg l.w.). In group 2, each animal received 1g/10 kg of l.w. of pellets of the fungus *M. thaumasium* (0.2g of fungus/10Kg l.w.), twice a week, for five months. In group 3 (control), the animals received 1g/10 kg of live weight of pellets without fungus. The monthly average of the egg countings per gram of feces of the animals of groups 1 and 2 treated, respectively were 71.6% and 61.1% smaller, respectivelly, in comparison to the animals of group 3 (control). The treatment of sheep with pellets containing the nematophagous fungi *D. flagrans* and *M. thaumasium* may be used as an alternative for the control of sheep gastrointestinal nematodiasis.

Keywords: Nematophagous fungi, *Duddingtonia flagrans*, *Monacrosporium thaumasium,* sheep, biological control.

Introduction

Sheep raising is a widely explored activity in tropical countries, seeking meat, milk and leather production (Vieira 2003). The management practices and technologies used are not always adequate, which leads to health problems, especially those related to helminthosis (Ahid et al. 2008).

The gastrointestinal parasitism by nematodes causes diseases and can kill ruminants throughout the world, but the greatest economic impact is in the growth decrease of young animals, resulting in low productivity (Araújo et al. 2007). Younger sheeps are more susceptible to helminthosis than adults and with increasing age acquires immunity against these parasites. All age groups are affected by helminths, but the most susceptible to this helminthosis are the lambs, and pregnant females (Urquhart et al. 1998).

The severity of the disease and the losses in production and productivity depend on the intensity of the infection, immunity and the host nutritional status (Stear et al. 2007). It is very important to understand the epidemiology of the ruminant helminthosis in order to provide an efficient strategic control in a region.

The indiscriminate use of antihelminthic provoked the selection of helminthes populations that are resistant to the different chemical groups used in sheep treatments (Mota et al. 2003). Besides, the chemical residues of such products have a negative impact on the environment and public health (Boray et al. 1990).

The use of alternative control methods have been encouraged due to the antihelminthic resistance by gastrointestinal parasite nematodes all over the world (Waghorn et al. 2003; Stear et al. 2007). The use of fungi for worm control may be a promising alternative (Alves et al. 2003; Araújo et al. 2004, 2006). Nematode antagonist fungi spread in feces and produce specialized structures (traps) with the objective of capturing and fixing nematodes (Mota et al. 2003). After fixation, the fungus penetrates the prey, killing it by the destruction of its internal organs. Several species of nematophagous fungi, such as *Duddingtonia flagrans* and *Monacrosporium thaumasium*, have been used in the biological control of gastrointestinal helminthosis in animals (Gomes et al. 1999; Araújo et al. 2000). The capacity that these species

present in surviving the passage through the gastrointestinal tract of ruminants without losing their predatory activity is an important pre-requisite in the biological control of helminthosis (Araújo and Sampaio, 2000; Dias et al. 2007).

Sodium alginate-based formulations have been evaluated experimentally in the control of animal parasite nematodes by some research laboratories. Such formulations have provided good results under laboratory and field conditions (Campos et al. 2007).

The objective of this work was to evaluate the effectiveness of the nematophagous fungi *D. flagrans* and *M. thaumasium,* by testing pellets formulations of these fungi in a sodium alginate matrix, in the biological control of gastrointestinal nematodiasis in sheep raised in the field.

Material and Methods

Organisms

Panagrellus sp. (free-living nematodes) were kept in Petri plaques with a medium of dampened and mashed oat flakes. These nematodes were removed from the culture medium through the immersion of small amounts of oat in distilled water in the Baermann funnel and collected in tubes of hemolysis after 6 hours of decantation. The nematodes were counted, by means of light microscopy (40x), by taking six aliquots of 10 µL, taking the average and extrapolating for the total volume.

Two isolates of the predatory fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were kept at 4ºC, in the dark and in test tubes containing cornmeal-agar 2%. These isolates came from a Brazilian soil and belonged to the mycology collection of the Universidade Federal de Viçosa.

To induce the formation of the fungal mycelium, culture discs of approximately 5 mm were transferred for 250 mL Erlenmeyers flasks containing 150 mL of potato-dextrose (Difco) liquid medium, pH 6.5, under the agitation of 120 rpm, in the dark and at the temperature of 26^{o}C, for 10 days. After this period, the mycelium was removed, with the use of a platinum loop, and weighed in an analytic scale for the future production of the pellets,

which were made in a sodium alginate matrix, according to Walker and Connick (1983) and modified by Lackey et al. (1993).

In vivo experimental essay

The experiment was developed in the experimental farm of the Universidade Federal de Viçosa, located in the city of Canaã, state of Minas Gerais, southeast of Brazil, latitude 20º45'20" and longitude 42º52'40", from May to October 2007.

In the beginning of the experiment, 24 Santa Inês sheep, at the ages from 15 to 48 months, with average l.w. of 32.5 kg were previously treated with a dose of Trimix®-Merial from Brazil, [closantel (10%), albendazole (5%), levamisole (6.4%), ivermectina B1a (0.2%), selenium (0.1g) and cobalt (0.44%)], with the oral dose of 1ml/10Kg of l.w.

After the 15th day of the antihelminthic treatment, the sheep were placed into three groups 1, 2 and 3, each of them with eight animals. The characteristics considered for this division were the weight and age similarity between the animals of the groups. Later, each group was placed in three pickets, each one with 2.0 ha and pasture of *Brachiaria decumbens*, naturally infested with gastrointestinal parasite helminths, due to the previous grazing of young and adult animals. In group 1, each animal received 1 gram of pellets (0.2 g of fungal mycelium) for each 10 kg of l.w. containing the fungus *D. flagrans* (AC 001). In group 2, each animal received 1 gram of pellets (0.2 g of fungal mycelium) for each 10 kg of l.w. containing the fungus *M. thaumasium* (NF34a). In group 3, the animals received 1 gram of fungus-free pellets for each 10 kg of l.w. All the animals received the pellets orally, twice a week, mixed in concentrated and balanced ration, provided daily for meat sheep (22% of total protein – Universidade Federal de Viçosa), and water *ad libitum* during five months, starting from June 2007.

After the introduction of the sheep in the pasture, samples of feces were collected, twice a week, directly from the rectal ampulla, determining the egg counting by gram of feces (EPG), according to Gordon and Withlock (1938). Every day, meteorological data were recorded, in a specialized station in the region, referring to the averages of the maximum, average and minimum monthly temperatures and monthly air relative humidity and rainfall.

Samples of feces were collected from the animals to observe the growth of the fungi. The feces were placed in plaques containing water-agar 2% and 1.000 *Panagrellus* sp. and put into a greenhouse, at 25°C, for 10 days.

Simultaneously to the EPG exam, coprocultures were carried out, for each animal, according to the methodology described by Roberts and O´Sullivan (1950). The identification of the infectant larvae in the coprocultures was performed according to Keith (1953).

EPG count curves (EPG) and larvae recovered from coprocultures of animals of both treated and control groups were recorded, and percentage of larval reduction was determined according to Mendoza-De-Guives et al. (1999):

$$\text{reduction (\%)} = \frac{\text{Mean L3 recovered from control group - Mean L3 recovered from treated group}}{\text{Mean L3 recovered from control group}} \times 100$$

Every 15 days, in each picket of the several groups of animals, two samples of pasture were collected, in W of varied points, alternates of up to 20 cm from the fecal mass and 20-40 cm far from the fecal mass, according to Amarante et al. (1996). Then, 500g of pasture were weighed, and larvae of sheep parasite nematodes were recovered from there. The pasture samples were put into a drying greenhouse at 100°C, for 3 days, for the achievement of dry matter. The data obtained were transformed in number of larvae by kilogram of dry matter.

Every 15 days, the scores of color intensity of the ocular mucosa were achieved in the animals, by the Famacha method, according to Bath and Van Wick (2001).

The weather conditions, such as temperature, air relative humidity and rainfall are represented in Figure 3.

The EPG originated from the coprocultures, number of infective larvae recovered from paddocks (L3), correlation between EPG and recovered L3 were compared over the experimental period. Weight of the animals and the scores achieved by the Famacha method were compared during the months of the experiment, starting from June 2007. Data were transformed into log (x + 1) and then examined by analyses of variance (ANOVA) and Tukey's

multiple comparison test with 1% probability. The analyses were performed using the BioEstat 3.0 Software (Ayres et al., 2003).

Results

The monthly average values of the EPG countings are represented in Figure 1. In the beginning of the experiment (June), the EPG of the animals in group 1 was higher than that of the animals in groups 2 and 3, but no statistical difference was observed ($p>0.05$). From the month of July, the animals in group 3 (control) presented higher averages for the EPG than the animals in groups 1 and 2, until the end of the experiment (October). On the other hand, in the last three months of the experiment (August, September and October), a statistical difference was observed ($p<0.05$) between the animals in groups 1 and 2 and the animals of the control group. There was no difference ($p>0.05$) between the animals in groups 1 and 2 treated with the fungi *D. flagrans* and *M. thaumasium,* respectively, during the 5 months of the experiment. Besides, the monthly average of the EPG of the animals in group 1 treated with pellets containing the fungus *D. flagrans* was 71.6% lower, compared to the animals in group 3 (control). For the animals in group 2 treated with pellets containing the fungus *M. thaumasium,* the reduction observed was of 61.1%, in comparison to the animals in group 3 (control). This decrease for the percentage values found for the animals in groups 1 and 2, was probably determined by the action of the fungi *D. flagrans* and *M. thaumasium*, also considering that this action was satisfactorily demonstrated in the last 3 months of the experiment.

The percentage values corresponding to the infectant larvae (L3) recovered from the coprocultures are represented in Fig. 2. A difference ($p<0.01$) was observed in the percentages of *Haemonchus* sp. of the animals in group 1 and 2 in relation to the animals in group 3, only in September. *Haemonchus* sp. was the most prevailing gastrointestinal parasite nematode in the experiment in all the groups, with percentages of 77.85%, 92.86% and 100% for the animals of the groups 1, 2 and 3 respectively, in the last month of the experiment (October). For the other

gastrointestinal nematodes (*Cooperia, Oesophagostomum.* and *Trichostrongylus),* no difference (*p>0.05*) was observed during all the experiment between the animals of the three groups and they were presented in very low levels.

As to the number of L_3 of *Cooperia* sp., *Haemonchus* sp., *Oesophagostomum* sp. and *Trichostrongylus* spp. recovered from the paddocks in the distances of up to 20 cm from the fecal mass and 20-40 cm far from the fecal mass, no difference (*p>0.05*) was observed between the groups during all the experiment (from June to October). For the group 1, in the distances of up to 20 cm and 20-40 cm from the fecal mass, the reduction percentual was 33.33% and 50%, respectively, in relation to group 3. Group 2 showed percentual reduction of 33.33% and 47.22%, at the same distances, in relation to group 3.

As to the weight of the animals, it was not observed any difference (*p>0.05*) between the animals from the three groups during all the experiment.

The Famacha method revealed a difference between the animals in groups 1 and 2 (*p<0.05*) and the animals in group 3 in August and September.

Discussion

In the present work, the animals in group 1 treated with the fungus *D. flagrans* presented a reduction of 71.6% in the EPG, compared to the animals in group 3 (control). Such results are in accordance with Sanyal et al. (2008), who also recorded a decrease in the EPG for the animals treated with this fungus. In another work, Dimander et al. (2003) also demonstrated the effectiveness of the application of *D. flagrans* in the control of ruminant gastrointestinal nematodiasis. Several works using the fungus *D. flagrans* in ruminants also recorded smaller monthly average values of the EPG counting in the treated animals, compared to the control group (Knox and Faedo 2001; Fontenot et al. 2003; Araújo et al. 2004; Paraud et al. 2007).

For the animals of group 2 treated with *M. thaumasium*, this reduction was of 61.1% in comparison to the animals in group 3. Fungi of the genus *Monacrosporium* sp. (*M. sinense, M. thaumasium, M. appendiculatum, M. ellypsosporum*) were evaluated by several authors, demonstrating effectiveness in the control of gastrointestintinal nematodiasis of different animal species (Araújo et al. 1992; Gomes et al. 1999; Castro et al. 2003; Assis and Araújo 2003; Campos et al. 2006; Araújo et al. 2007). These results are also in accordance with the present work, since the EPG decrease was observed in the animals in the group treated with *M. thaumasium*.

In the coprocultures, *Haemonchus* sp. was the most prevailing gastrointestinal parasite nematode in all the groups, reaching percentages of 77.85%, 92.86% and 100% for the animals in groups 1, 2 and 3, respectively, in October. Such results agree with Amarante et al. (2004) and Rocha et al. (2008), who demonstrated that in the southeast region of Brazil, *Haemonchus contortus* and *Trichostrongylus colubriformis* were the most prevailing gastrointestinal nematodes in sheep. *H. contortus* was also the most prevailing gastrointestinal nematode in goats, followed by *T. colubriformis*, in the Brazilian semiarid region (Araújo et al. 2007). These results are also in accordance with the present work (Figure 2).

In Malaysia, Chandrawathani et al. (2002) observed the effectiveness of the treatment with the fungus *D. flagrans*, daily administered to ovine. On the other hand, Terrill et al. (2004), using this same fungus, found a reduction in the larvae found in feces of goats infected mainly with *H. contortus*. According to Casillas et al. (2008), *D. flagrans* can be considered a viable alternative for the control of haemonchosis in sheep, which were treated with chlamydospores of this fungus. Araújo et al. (2007) also recorded a larva decrease in the coprocultures of the animals treated with the fungus *M. thaumasium* in the Brazilian semi-arid. These results are in accordance with the present work, where it was observed the effectiveness of the fungi *D. flagrans* and *M. thaumasium* on sheep gastrointestinal parasite nematodes.

The number of (L_3) recovered from the paddocks in the distances of up to 20 cm from the fecal mass and 20-40 cm far from the fecal mass was similar ($p>0.05$) for the animals in groups 1, 2 and 3 in the months when the

experiment took place. However, Larsen et al. (1998) evaluated the potential of the fungus *D. flagrans* in the control of the free living stages of the animal parasites and achieved a reduction of more than 80% of the number of L3 in paddocks.

The correlation coefficient between EPG and infective larvae recovered from paddocks of group 1 within 0–20 cm from fecal pats was 0.7262; and for the distance 20–40 cm was 0.8076. For group 2, the correlation coefficient between EPG and infective larvae recovered within 0–20 cm from the fecal pats was -0.5760 and within 20–40 cm was 0.2436. For group 3, the correlation coefficient recovered within 0–20 cm from the fecal pats was -0.7519 and within 20–40 cm was -0.3864. In group 1, was observed strong positive correlation between EPG and infective larvae. In group 2, these results showed negative correlation within 0–20 cm and positive within 20–40 cm. Although in the group 3 was observed negative correlations between EPG and infective larvae. Dias et al. (2007) pointed out, there might be dependence between EPG and infective larvae recovered from pastures even if the correlations are null. Besides, the availability of larvae on pasture may be determined by contamination from animals, as well as environmental factors, parasite and host (Lima et al., 1997).

Weather conditions, such as temperature, air relative humidity and rainfall were responsible for the development of the free living stages of the nematodes in paddocks, since they were observed in the pickets of all groups and in all the months. According to Honer and Bianchin (1987), during the rainy season, pasture contamination would reach maximum levels, while in the dry season, the helminth population would survive almost exclusively inside the hosts, due to the less favorable weather. These results agree with the present work, in which the highest recovery of larvae from paddocks was observed in the periods with higher rainfall indices (August and September; Fig. 3).

It was not observed any difference ($p>0.05$) in the weight gain of the animals of group 1 treated with pellets containing the fungus *D. flagrans* and the animals of group 2 treated with pellets containing the fungus *M. thaumasium*, in comparison to the animals in group 3 (control). On the other hand, in Malaysia, Chandrawathani et al. (2004) achieved a better weight

gain for the animals (Santa Inês x Corriedale) treated with the fungus *D. flagrans* in an experiment carried out in the field during approximately one year. A higher weight gain in the animals was also observed by Araújo et al. (2007) when testing the fungus *M. thaumasium* in goats in the Brazilian semi-arid.

In the present work, the application of the Famacha method in the animals revealed difference ($p<0.05$) between the animals in groups 1 and 2 and the animals in group 3 only for August and September. Molento (2004) did not observe a satisfactory result either, when using this method along with the chemical control in small ruminants.

The nematophagous fungi *D. flagrans* and *M. thaumasium* used in the present work were responsible for the EPG decrease of the animals in groups 1 and 2, in comparison to the animals in group 3.

Conclusion

The treatment of sheep with pellets containing the nematophagous fungi *D. flagrans* and *M. thaumasium* can be used in the control of sheep gastrointestinal nematodes.

Acknowledgements

This study was supported by the Research Project of the Universidade Federal de Viçosa, Brasil. Thanks are also due to FAPEMIG for the financial support and CAPES for the scholarships granted.

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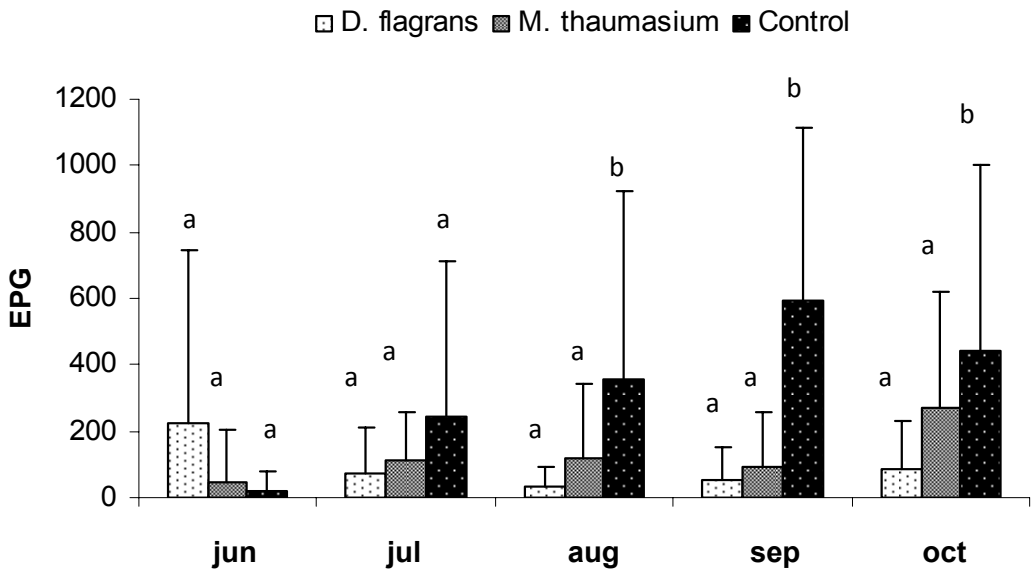

Figure 1 – Monthly averages and standard deviations of the countings of eggs per gram of feces (EPG) of the animals in the groups treated with the nematophagous fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* (0,2g of fungus/10Kg of live weight) and the control group, collected from June to October 2007, Viçosa, Minas Gerais, Brazil. The values followed by the same letters (a) are similar (*p>0.05*) – Tukey Test.

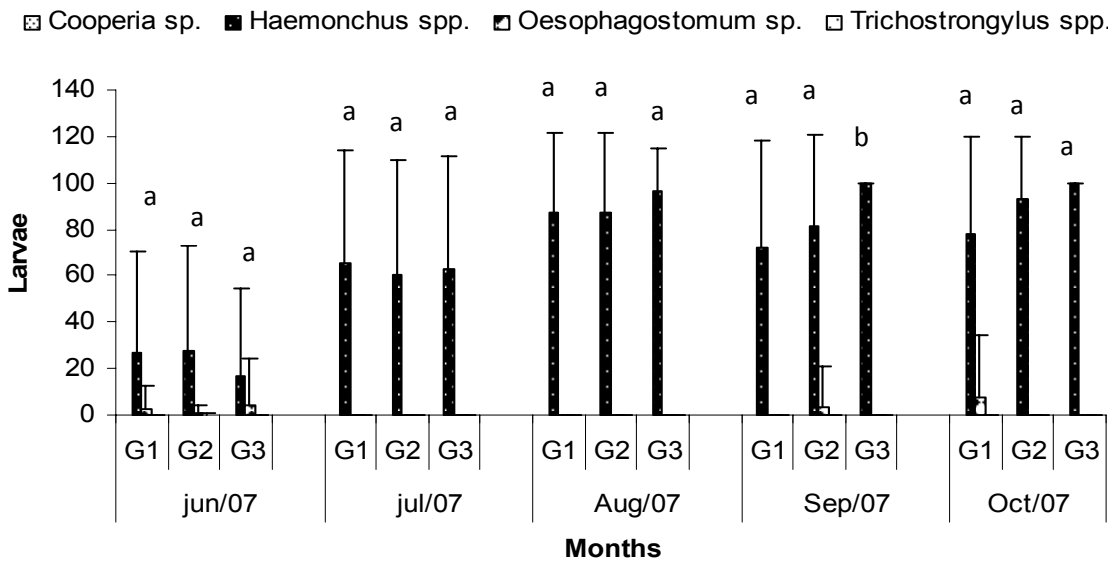

Figure 2 – Monthly average values and standard deviations of the larvae of gastrointestinal nematodes (*Cooperia* sp., *Haemonchus* spp., *Oesophagostomum* sp., *Trichostrongylus* spp.) recovered from the coprocultures of the animals in the groups treated with the fungus *Duddingtonia flagrans* (G1) and the fungus *Monacrosporium thaumasium* (G2) (0.2g de fungus/10Kg of live weight) and the control group without fungus (G3), collected from June to October 2007, Viçosa, Minas-Gerais, Brazil. The values followed by the same letters (a) are similar (*p>0.05*) – Tukey Test.

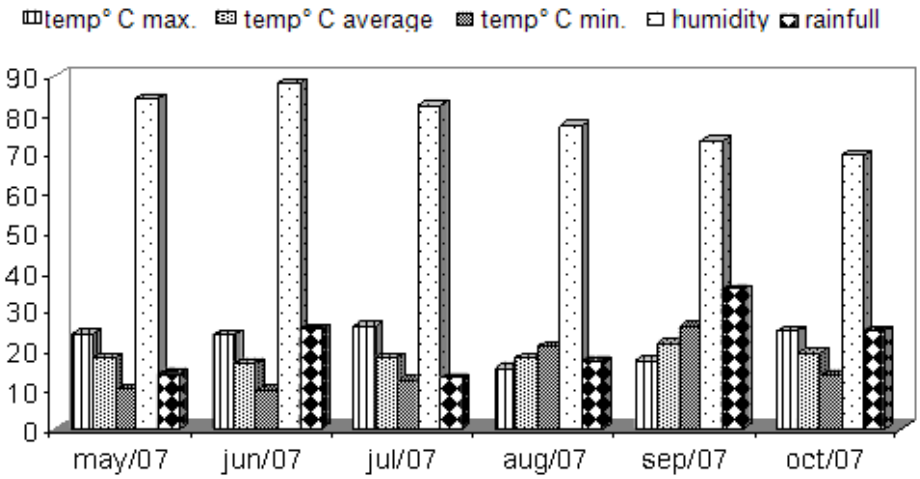

Figure 3 – Monthly average temperature; minimum, average and maximum temperature (ºC) temperature, air relative humidity (%) and rainfall (mm^3) recorded from May to October 2007, Viçosa, Minas Gerais, Brazil.

(ii) Capítulo 2

Activity *in vitro* of fungal conidia of *Duddingtonia flagrans* and *Monacrosporium thaumasium* on *Haemonchus contortus* infective larvae

Journal of Helminthology, V.21, p. 1-4, 2010.
DOI: 10.1017/S0022149X10000362
Published online: 21 July 2010

Abstract

The application of biological control with nematophagous fungi is a viable alternative. The objective of this work was to evaluate the predatory activity of *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34a) on *Haemonchus contortus* infective larvae (L_3) in two experimental assays (A and B). In the assay A, two treatments and one control were formed and kept for seven days in Petri dishes with 2% water-agar. Each treatment consisted in 1000 *H. contortus* L_3 and 1000 conidia of only one fungal isolate, and the control group consisted on 1000 L_3, without fungus, with 10 repetitions per group. In the assay B, 1000 conidia of one of the fungal isolates, AC001 or NF34a, were added to coprocultures made from 20g of feces collected from sheep naturally infected with *H. contortus*. At the end of the experiment, the Baermann method was used to the count the non-predated larvae of all Petri dishes from treatment and control groups. In the assay A, no difference was observed ($P > 0.05$) between the groups treated with AC001 and NF34a fungi. A difference was observed ($P < 0.05$) between the treated and control groups. The L_3 reduction percentage at the end of the experiment were 87.75% and 85.57%, respectively, for the fungal isolates compared to the control group. In assay B, the reduction percentages for conidia of these isolates were 85.82% and 87.32%, respectively. *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were effective in the *in vitro* control of sheep *H. contortus* L_3 and could be used in the biological control of this nematode.

Introduction

Significant economic losses in sheep production are associated with the parasitism by gastrointestinal helminths, mainly *Haemonchus contortus*. The annual world economic losses due to infections caused by gastrointestinal nematodes are estimated in millions of dollars (Amarante *et al*., 2009; Silva *et al*., 2009). In addition, the frequent use of anthelmintics for the prophylaxis of gastrointestinal nematode (GIN) infections has led to the dissemination of populations of resistant parasites (Getachew et al., 2007). Researches worldwide looks for alternative measures for control of domestic animals helminthiasis, aiming to reduce the usage of chemotherapeutics (Silva *et al*., 2009). The application of biological control using nematophagous fungi has become a viable alternative and has presented itself as promising. Some work done with the species *Duddingtonia flagrans* and *Monacrosporium thaumasium* demonstrate their effectiveness in the control of nematodes in the laboratory and field (Waller *et al*., 1994; Larsen, 1999; Terril *et al*., 2004). The objective of this work was to evaluate *in vitro* activity of fungal conidia of *D. flagrans* and *M. thaumasium* on *H. contortus* infective larvae.

Material and methods

Fungal culture and experimental assays

The isolates of the predator fungi species *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were kept in test tubes containing 2% corn–meal–agar (CMA) in the dark, at 4°C for 10 days. The isolates were previously stored at the Laboratory of Parasitology in the Department of Veterinary Medicine, Federal University of Viçosa, Minas Gerais, Brasil. Petri dishes containing 2% water-agar (2% WA) were inoculated with one of the isolates and incubated at 26°C for ten days. After that, the conidia were collected according to Araújo *et al*. (1993).

The present work consisted in two experimental assays (A and B). In the assay A, the predatory activity of the predator fungi *D. flagrans* and *M. thaumasium* on *H. contortus* L_3 was evaluated in an *in vitro* assay on Petri dishes containing 2% WA. In the assay B, the predatory capacity of the

fungal conidia of the isolates *D. flagrans* and *M. thaumasium* on sheep faeces naturally infected by *H. contortus* was evaluated.

Collection of L_3 *Haemonchus contortus*

The *H. contortus* L_3 were obtained by the Baermann method after coprocultures were carried out for 10 days, according to Gordon & Whitlock (1939), using naturally infected sheep faeces that were positive for superfamily Strongyloidea. Using light microscopy (x10 objective lens) the samples with 100% of *H. contortus* were used in the experiment, having been washed with distilled water and centrifuged five times to remove the supernadant. The coprocultures were carried out, for each sheep, according to the methodology described by Roberts & Sulivan (1950). The identification of the infective larvae in the coprocultures was performed according to Keith (1953).

Collection of conidia

Culture discs (4mm in diameter) were removed from the fungal isolates kept in test tubes containing 2% CMA and transferred to 9.0-cm Petri dishes containing 20 ml of 2% potato dextrose agar and kept at 25°C in the dark for 10 days. After growth, new culture discs (4 mm in diameter) were transferred to 9.0-cm diameter Petri dishes containing 20 ml of 2% water-agar (2% WA) and 1ml of distilled water containing 1000 larvae of *Panagrellus* sp. was added daily for 21 days for induce conidia formation. When fungal development was complete, 5ml of distilled water were added to each Petri dish, and the conidial and mycelia fragments were removed as described by Araújo *et al*. (1993).

Assay A

Two treatments and one control were formed and kept at 26°C for 7 days. Each treatment consisted in 1000 conidia of a fungal isolate and 1000 *H. contortus* L_3 with 1000 L_3 without fungus as the control, and ten repetitions per group. Ten random fields of each plate were counted daily, checking the number of predated larvae until the end of the experiment (seventh day).

After the last reading of the plates, the Baermann method was used for counting the non-predated larvae of all treated groups and control plates.

Assay B

In coprocultures made from 20g of faeces from sheep naturally infected by *H. contortus*, 1000 conidia of the fungal isolates *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were homogeneized and plated. In the control group, only coprocultures made from 20g of faeces from sheep naturally infected by *H. contortus* were used. There were six replicates per group. At the end of 7 days, the *H. contortus* larvae were recovered from the treated and control groups using the Baermann method, according to the technique described by Araújo *et al*. (1993).

Data analysis

Means of recovered *H. contortus* L_3 were calculated. Data were examined by analysis of variance at significance levels of 1 and 5% probability (Ayres *et al*., 2003). Predation efficiency of L_3 relative to the control group was evaluated by the Tukey's test at 5% probability. The reduction percentage of L_3 means was calculated according to the following equation: Reduction%= (Mean of L_3 recovered from control – Mean of L_3 recovered from treatment)/Mean of L_3 recovered from control x 100.

Results and discussion

The mean of non-predated *H. contortus* L_3 per 4mm diameter field during the experiment is shown in table 1. The *D. flagrans* (AC001) and *M. thaumasium* (NF34a) fungal isolates predated the larvae throughout the experiment assay. The predation was observed on the first reading of the two treated groups, 24 hours after the interaction between the larvae and the fungal isolates. However, the presence of fungus and formed traps predating the L_3 were not observed in the control group Petri dishes.

At the end of the experimental assay, no difference was found ($P > 0.05$) between the groups treated with the fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34a), nonetheless, difference were observed between the

treated and the control groups (fig. 1). The reduction percentages of the L_3, obtained using the Baermann method at the end of the experiment, with the fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34a), were 87.75% and 85.57%, respectively, compared to the control group.

At the end of 7 days, the conidia of the *D. flagrans* (AC001) and *M. thaumasium* (NF34a) isolates reduced the number of recovered L_3 from Assay B. However, no difference ($P < 0.05$) was found between the treatments. Moreover, difference ($P < 0.05$) was observed between the treatments and the control group (fig. 2). The reduction percentage due to the *D. flagrans* (AC001) and *M. thaumasium* (NF34a) isolates were 85.82% and 87.32%, respectively.

In the presence of nematodes, the fungi are able to produce traps in a short period of time. This predation was already seen in the first reading of the Petri dishes of the treated groups 24 h after the interaction between the larvae and the fungal isolates. However, the presence of fungus and of formed traps predating the L_3 present in the control group Petri dishes was not observed.

During the present work, the temperature was kept constant at 26°C, and it was observed that there was an effective reduction of the number of *H. contortus* L_3 due to the presence of *D. flagrans* and *M. thaumasium* fungal isolates throughout and after 7 days of interaction (table 1 and fig. 1). According to Gronvold *et al*. (1996), after the capture of the larvae by the trapping structures of the predatory fungi, a process of hyphae penetration into the cuticle occurs, followed by the digestion of the nematodes' interior. The most commonly observed differentiated structures of these fungi along the mycelium are non-adhesive constricting rings, nonconstricting rings, buttons, and three-dimensional adhesive networks. These results are in accordance with the present work, since the production of traps on the *H. contortus* L_3 was observed in the experimental assay.

Furthermore, *M. thaumasium* (NF34a) destroyed the *H. contortus* L_3, with a reduction of 85.57% of the larvae at the end of the experimental assay, demonstrating its effectiveness. Fungi of the genus *Monacrosporium* (*M. sinense* and *M. thaumasium*), have been evaluated by several authors,

demonstrating effectiveness in the control of gastrointestinal nematodioses of different animal species (Araújo *et al*., 1992; Braga *et al*., 2009).

In the assay B, a reduction of the number of *H. contortus* L_3 recovered from the faeces was observed, caused by conidia of *D. flagrans* (AC001) and *M. thaumasium* (NF34a) isolates. These results are in accordance with Silva *et al*. (2009), who also observed that these isolates could be used in the control of sheep haemonchosis in natural conditions. In the same work, *H. contortus* was the most prevalent gastrointestinal parasite nematode, demonstrating its importance in sheep production.

The fungal conidia of *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were effective *in vitro* control of sheep *H. contortus* infective larvae (L_3) and could be used in the biological control of this nematode.

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Table 1. Daily means and (±) standard error of non-predated infective larvae (L_3) of *Haemonchus contortus* per 4mm diameter field in 2% water-agar during seven days of treatment with the fungal isolates *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34a) and control without fungus.

Time (Days)	Treatment (Mean of L_3 non-predated)		
	AC001	NF34a	Control
1	1.33 ± 1.93^{A}	1.83 ± 2.05^{A}	5.85 ± 5.83^{B}
2	0.77 ± 1.20^{A}	1.8 ± 1.59^{A}	5.07 ± 4.72^{B}
3	0.65 ± 0.95^{A}	4.85 ± 2.78^{B}	15.1 ± 6.92^{C}
4	1.23 ± 1.83^{A}	6.87 ± 3.09^{B}	22.53 ± 7.69^{C}
5	0.83 ± 1.55^{A}	0.78 ± 0.76^{A}	12.1 ± 5.96^{B}
6	0.58 ± 1.06^{A}	0.43 ± 0.56^{A}	9.92 ± 4.83^{B}
7	0.25 ± 0.47^{A}	0.02 ± 0.13^{A}	14.88 ± 6.30^{B}

Means followed by same letter in the same line are not significantly different ($P > 0.05$) — Tukey test.

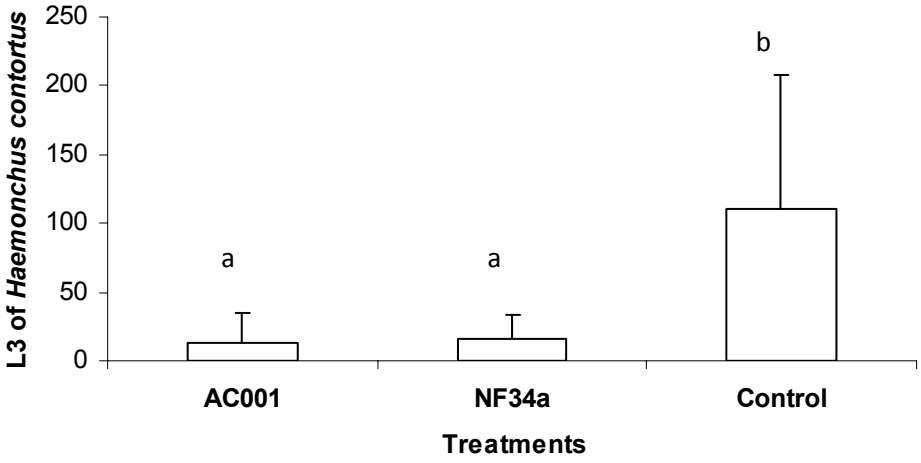

Fig. 1. Mean number of non-predated *Haemonchus contortus* infective larvae (L_3) recovered in 2% water-agar by the Baermann method on the seventh day after interaction with the fungal isolates *Duddingtonia flagrans* (AC001*), Monacrosporium thaumasium* (NF34a) and control (without fungus). Lines on bars represent standard deviations. Means accompanied by at least one common letter (a) row are not significantly different by Tukey's test at a 5% probability level.

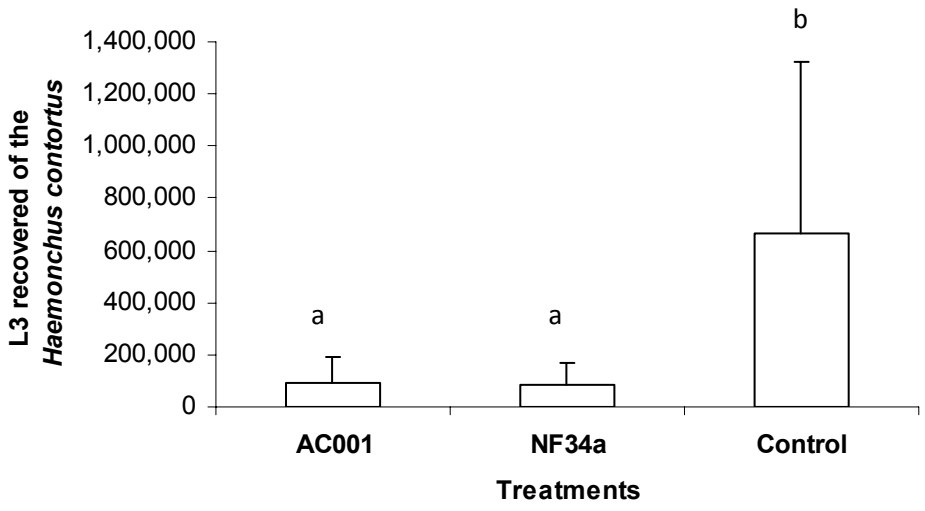

Fig. 2. Mean number of non-predated *Haemonchus contortus* infective larvae (L_3) recovered in coprocultures by the Baermann method on the seventh day after interaction with the fungal isolates *Duddingtonia flagrans* (AC001*), Monacrosporium thaumasium* (NF34a) and control (without fungus). Lines on bars represent standard deviation. Means accompanied by at least one common letter (a) are not significantly different by Tukey's test at a 5% probability level.

(ii) Capítulo 3

Comparative analysis of destruction of the infective forms of *Trichuris trichiura* and *Haemonchus contortus* by nematophagous fungi *Pochonia chlamydosporia, Duddingtonia flagrans* and *Monacrosporium thaumasium* by scanning electron microscopy

Veterinary Microbiology, 147 (2011) 214-219
DOI: 10. 1016/j.vetmic.2010.06.019
Available online 1 July 2010.

Abstract

Nematophagous fungi are a viable and promising alternative, and or an option to control gastrointestinal nematodes of humans and domestic animals. The present study aimed to demonstrate by scanning electron microscopy (SEM) the *in vitro* predatory activity of nematophagous fungi *Pochonia chlamydosporia* (VC1 and VC4 isolates) *Duddingtonia flagrans* (AC001 isolate) and *Monacrosporium thaumasium* (NF34a isolate) on eggs of *Trichuris trichiura* and infective larvae (L3) of *Haemonchus contortus*. The work was divided into two experimental tests (A and B). In test A and B, the predatory activity of nematophagous fungi *P. chlamydosporia, D. flagrans* and *M. thaumasium* on eggs of *T. trichiura and H. contortus L3* was observed. After six hour, in test A, isolates *P. chlamydosporia* (VC1 and VC4) had a role in destroying eggs of *T. trichiura*. For fungi *D. flagrans* and *M. thaumasium* the ovicidal activity on *T. trichiura* eggs was not observed. Test B showed that *D. flagrans* (AC001*)* and *M. thaumasium* (NF34a) were capable of predating *H. contortus* L_3, but no predation by the fungus *P. chlamydosporia* was seen. These fungi can be offer potential for the biological control of nematodes.

Key-words: *Trichuris trichiura*; *Haemonchus contortus*; *Duddingtonia flagrans*; *Monacrosporium thaumasium*; *Pochonia chlamydosporia*; biological control.

1- Introduction

Alternative measures for the control of endoparasitosis of humans and domestic animals have been widely searched in the world by researchers (Braga et al., 2007; Araújo et al., 2008). *Trichuris trichiura* is a nematode of major importance in public health, infecting approximately 604 million people in the world, being the second greatest prevalent globally. In mild infestations, this nematode causes few symptoms, but in massive infestations it can cause bloody diarrhea and diarrhea with mucus, and it can be associated with rectal prolapse. In the developing countries of Asia, Africa and Latin America the deficient sanitary infrastructure and hot, humid climates provide the necessary conditions for eggs of this parasite to incubate in the soil (Hotez et al., 2008).

Haemonchus contortus is a hematophagous nematode of major prevalence and veterinary medical importance for small ruminants in tropical climate countries, having as infective form a third stage larvae (L3) (Amarante et al., 2009).

Nematophagous fungi are a viable and promising alternative, which can be an option in controlling gastrointestinal nematodes of humans and domestic animals (Larsen et al., 1998; Braga et al., 2007). Its action is focused on faecal environments where important status changes of gastrointestinal nematode parasites take place. There are different types of nematophagous fungi, which can be characterized as opportunistic or parasitic of eggs, predators and endoparasites (Silva et al., 2009).

Among opportunists or parasites of eggs, the species *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*) stands out (Gams and Zaire, 2001). According to Lysek and Sterba (1991), the action of this fungus is based on appressorial formation, developed from undifferentiated hyphae, which allows the colonization of the egg surface and penetration through both mechanical and enzymatic actions, characterizing a type 3 effect (eggs destruction) (Lysek, 1976, 1978).

Species of predator fungi differ in their ability of capturing larvae of nematodes. They are the most studied organisms in the biological control of nematodes, and present higher potential for marketing (Gronvold et al., 1996). These fungi also show some kind of interaction on the eggs of

gastrointestinal helminthes parasites, but without causing its destruction. In this group, the *Duddingtonia* and *Monacrosporium* genera stand out due to their efficacy in the biological control of gastrointestinal nematode parasites (Dimander et al., 2003; Araújo et al., 2008).

The objective of this work was to demonstrate by scanning electron microscopy (SEM) the *in vitro* predatory activity of isolate fungi *Pochonia chlamydosporia, Duddingtonia flagrans* and *Monacrosporium thaumasium* on eggs of *T. trichiura* and infective larvae of *H. contortus*.

2- Material and methods

2.1. Fungi

Isolates of the nematophagous fungi of the species *P. chlamydosporia* (VC1) and (VC4), *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were maintained at 4° C, protected from light and in assay tubes with corn-meal-agar 2% (2% CMA). These isolates were further peaked for plates in water-agar medium 2% (2% WA) where they grew for seven days. Culture plates of 4 mm in diameter were extracted from fungal isolates maintained in assay tubes with 2% CMA and were transferred into Petri dishes of 9.0 cm in diameter with 20 ml of potato-dextrose-agar 2% and kept at 25º C, in the dark for 10 days.

2.2. Obtainment of *T. trichiura* eggs

T. trichiura unembryonated eggs were obtained from human faeces, descendents from Native Brazilian Amazon societies. They were extracted and concentrated by spontaneous sedimentation for 2-4hs, which were kept in refrigeration from 4 to 8º C. This material was donated by the Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil. The eggs were later washed with distilled water and centrifuged five times discarding the supernatant.

2.3. Obtainment of *H. contortus* infective larvae (L3)

H. contortus (L3) were obtained from coprocultures with 100% of *H. contortus* L3 from positive ovine faeces according to Gordon and Whitlock (1939). After stool tests at 26º C for ten days, L3 were recovered according

to Barçante et al. (2003), washed with distilled water and centrifuged five times discarding the supernatant.

2.4. Experimental assay and test A and B

This work is constituted by two experimental tests (A and B). In test A, the objective was to observe the ovicidal activity of fungi *P. chlamydosporia* (VC1 and VC4), *D. flagrans* (AC001) and *M. thaumasium* (NF34a) on eggs of *T. trichiura*. In test B, the objective was to observe the predatory activity of nematophagous fungi *D. flagrans* (AC001), *M. thaumasium* (NF34a) and *P. chlamydosporia* (VC1 and VC4) on L3 of *H. contortus*.

After the mycelial grew on the entire surface of the Petri dishes, 100 eggs of *T. trichiura* (test A) and 1000 *H. contortus* of L3 (test B) were poured on the dialysis membranes of the 9.0 cm in diameter Petri dishes with medium 2% WA of treated groups, and in control plates (without fungus). In the first 24 hs after the inoculation of nematodes on the Petri dishes, cultures on the plates were observed every six hour through a light microscope (100 X). After observing larvae of *H. contortus* larvae preyed and *T. trichiura* eggs in certain areas of the plate, marks were made on bottom of them with a permanent marker.

2.5. Scanning electron microscopy

Culture plates of fungal isolates *P. chlamydosporia* (VC1 and VC4), *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were transferred into disposable Petri dishes of 6.0 cm x 1.0 cm. Surfaces of the plates were covered with cellulose membrane plates (dialysis membrane), with proteins of a molecular weight over 12,000 Da and with a filtering capacity approx. 640 mL/ft (Sigma-Aldrich®, U.S.A). The dialysis membrane was cut into 6 cm diameter disks that were placed in Erlenmeyer flasks with distilled water. The material was autoclaved at 120º C for 15 min. After, they were removed from the Erlenmeyer flasks with the aid of a clamp and placed on 2% WA surfaces so that the membrane margins covered all agar surfaces and the edges were attached to the edge of the plates. These edges were also covered with 2% WA. Next, plates were incubated in the dark, at a temperature of 25º C for seven days (Nordbring-Hertz, 1983).

After 6 hs of observation of the interaction with *T. trichiura* eggs (test A) and with *H. contortus* L3 (test B), pieces of the dialysis membrane with eggs of *T. trichiura* parasitized fungi and L3 samples exposed to capture were cut with the aid of a blade, collected with a fine-tipped clamp and fixed in glutaraldehyde at 2.5% in 0.05 M of phosphate buffer, pH 7.4 and for 24 hs. Next, they were washed six times in the same buffer, post-fixed in osmium tetroxide 2% and dehydrated by passing the material in a series of ethyl alcohol (30, 50, 60, 70, 95 and 100%). The material was dry in critical point dryer BALZERS® using carbon dioxide, recovered with gold plating (Nordbring-Hertz, 1983; Guimarães and Caldeira, 1997) and electron-micrographed in a scanning electronic microscope LEO, model 1430VP at 10-15 kV.

3- Results

In the experimental test A, by observing SEM, it was found that *P. chlamydosporia* isolates (VC1 and VC4) had a role in destroying *T. trichiura* eggs after a 6 hour observation period (Figs. 1 a-b). The formation of appressoria of *P. chlamydosporia* on *T. trichiura* eggs caused the formation of a halo on the eggs surface, suggesting enzymatic and mechanic actions, later causing its penetration and destruction (Figs. 2 a-b).

For predator fungi *D. flagrans (*AC001*)* and *M. thaumasium* (NF34a), it was noticed by light microscope the interaction of predator fungi in the first 6 hour of the test with *T. trichiura* eggs where hyphae of these isolates were attached to the surface of eggs, without causing destruction was noticed by light microscope. Likewise, scanning electron microscopy revealed hyphae of *D. flagrans (*AC001*)* and *M. thaumasium* (NF34a) attached to the surface of the eggs during the observation period, but without destroying the eggs (Figs. 3 a and b). No predation of *T. trichiura* eggs in the control plates was observed (Fig. 4).

The results showed in test B that *H. contortus* L3 visualized by SEM, were preyed after 6 hour by the predator fungi *D. flagrans (*AC001*)* and *M. thaumasium* (NF34a) that produced traps and caused its destruction (Figs.5 a-c and 6). It was noticed in all Petri dishes that specific structures (conidia and traps) of predator fungi attached to nematodes and later caused its

destruction (Figs. 7 a-b). In addition, chlamydospores of fungus *D. flagrans* were observed on Petri dishes of the treated groups (Figs. 8 a-b). Concerning the fungus *P. chlamydosporia* (VC1 and VC4), no predatory activity on *H. contortus* L3 throughout the experimental test was noticed.

4- Discussion

During the experimental test A, it was noticed that *P. chlamydosporia* isolates (VC1 and VC4) were found to have a role in destroying *T. trichiura* eggs after the 6 hour observation period. This result is consistent with the work of Braga et al. (2008) who recorded by SEM the destruction of *Schistosoma mansoni* eggs throughout the experimental test. However, in the present work, the ovicidal activity of *P. chlamydosporia* was observed throughout the experimental test by SEM. This information is important, because its ovicidal action is characterized after 6 hour of interaction with the eggs. In addition, it was observed the formation of appressoria of *P. chlamydosporia* causing the penetration and destruction on *T. trichiura* eggs was observed in the present work. This is the first work analyzing the interaction of nematophagous fungi on *T. trichiuria* eggs.

In the experimental test B the production of traps, non-adhesive constricting rings, non-constrictive rings, adhesive 3D networks and buttons on *H. contortus* L3 was observed. Specific structures (conidia and chlamydospores) of fungus *D. flagrans* and *M. thaumasium* were observed attached to *H. contortus* L3. According to Araújo et al. (2004), the production of chlamydospores is the main requirement for a fungus to be used as a possible biological controller. For predator fungi *D. flagrans (*AC001) and *M. thaumasium* (NF34a), hyphae of these isolates attached to the surface of eggs using light microscope and SEM were noticed. These results are consistent with Braga et al. (2007) who mention that predator fungi show effect without causing the destruction of parasitized eggs. Concerning the fungus *P. chlamydosporia* (VC1 and VC4), it did not destroy *H. contortus* L3 throughout the experimental test. This information is consistent with works performed with this fungus on eggs of gastrointestinal helminthes (Braga et al., 2010) where its ovicidal action, and not larvicidal action, was proven.

Predator fungi formed traps produced at intervals throughout the hyphae. In pure cultures, many of these fungi did not form traps. The formation of these structures is a response to the presence of nematodes or their substances (Larsen, 1999). They are also induced by adverse culture conditions, such as scarcity of water and/or nutrients. The hyphae differentiation in this work occurred after the 6 hour interval where numerous trapping structures were produced. According to Araújo et al. (2004), after capturing larvae through capture structures of predator fungi a process of penetration of hyphae in the cuticle occurs, followed by the digestion of the internal content of the nematode. The most observed differentiated structures of these fungi throughout the mycelium are non-adhesive constricting rings, non-constrictive rings, adhesive 3D networks and buttons.

5- Conclusion

Using scanning electron microscopy, the results presented in this work showed that fungus *P. chlamydosporia* destroyed the *T. trichiura* eggs, showing for the first time its ovicidal activity. *D. flagrans* and *M. thaumasium* destroyed *H. contortus* L3. Therefore, these fungi could be used as possible biological controls of these nematodes.

Acknowledgments

The authors thank the Núcleo de Microscopia e Microanálise (NMM), of the Universidade Federal de Viçosa (UFV) and the Fundação Oswaldo Cruz (FIOCRUZ), Brazil. Thanks are also due to FAPEMIG, CAPES / FINEP and CNPq for the financial support.

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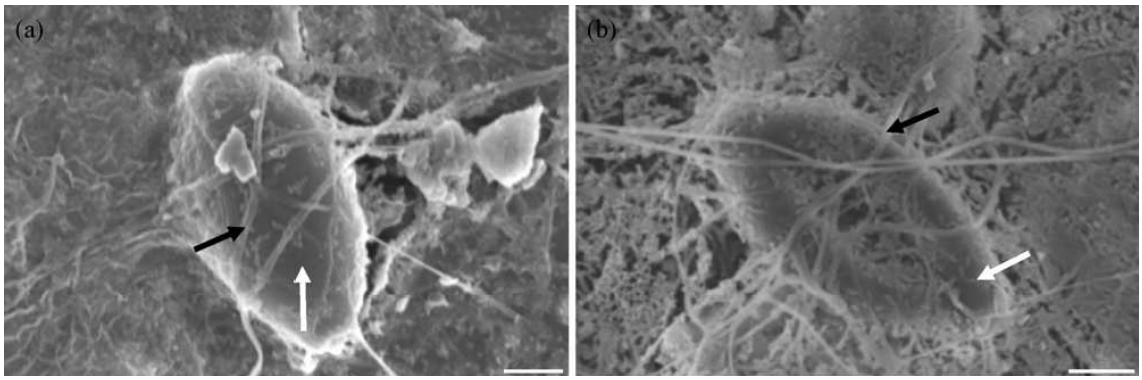

Fig. 1. (a and b) Destruction of *Trichuris trichiura* eggs (white arrow) after the 6-h observation period by isolates of *Pochonia chlamydosporia* (VC1 and VC4) (black arrow). SEM. Bar: (a) 10µmm; (b) 10µm.

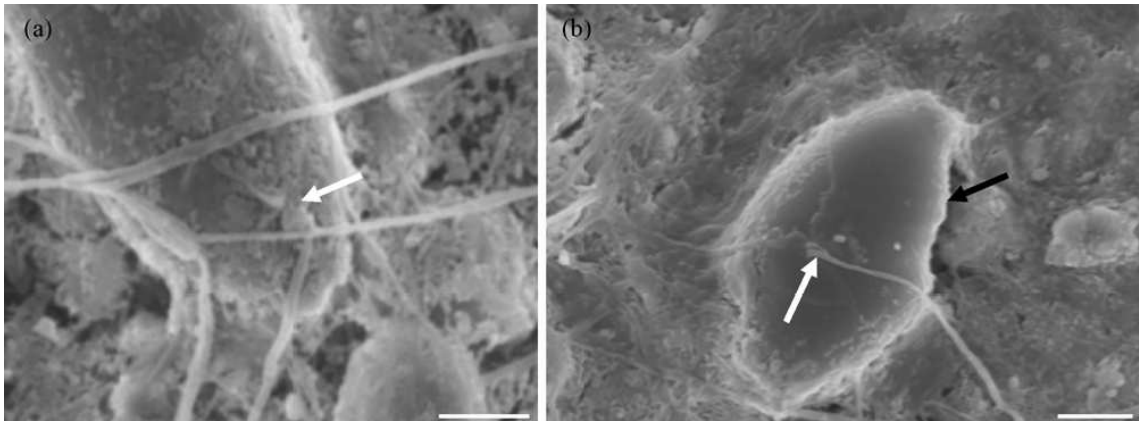

Fig. 2. (a and b) Formation of appressoria of *Pochonia chlamydosporia* (white arrow) on *Trichuris trichiura* eggs (a and b), causing its destruction (black arrow) (b). SEM. Bar: (a) 10µm; (b) 10µm.

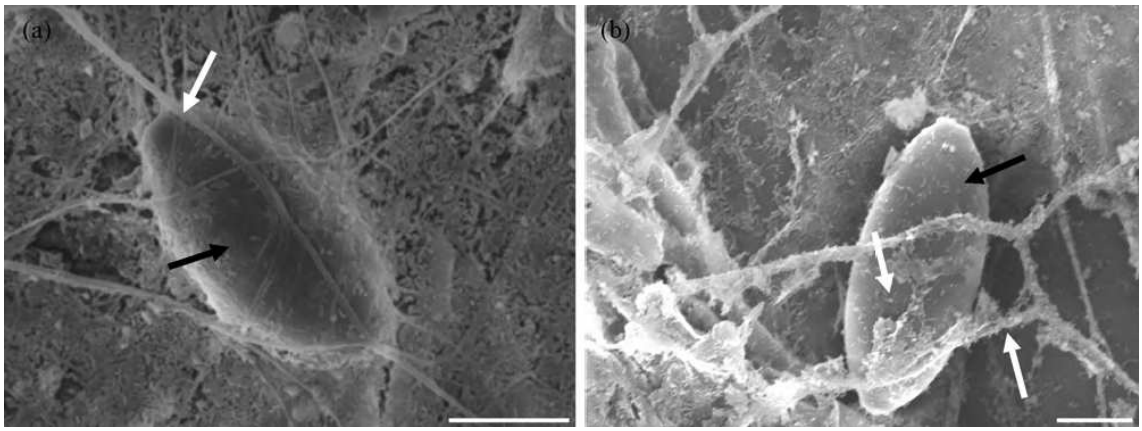

Fig. 3. (a and b) Hyphae of fungi (a) *Duddingtonia flagrans* (AC001) and (b) *Monacrosporium thaumasium* (NF34a) (white arrow) attached to the surface of eggs (black arrow) during the entire observation period, without destruction of eggs. SEM. Bar: (a) 20μm; (b) 20μm.

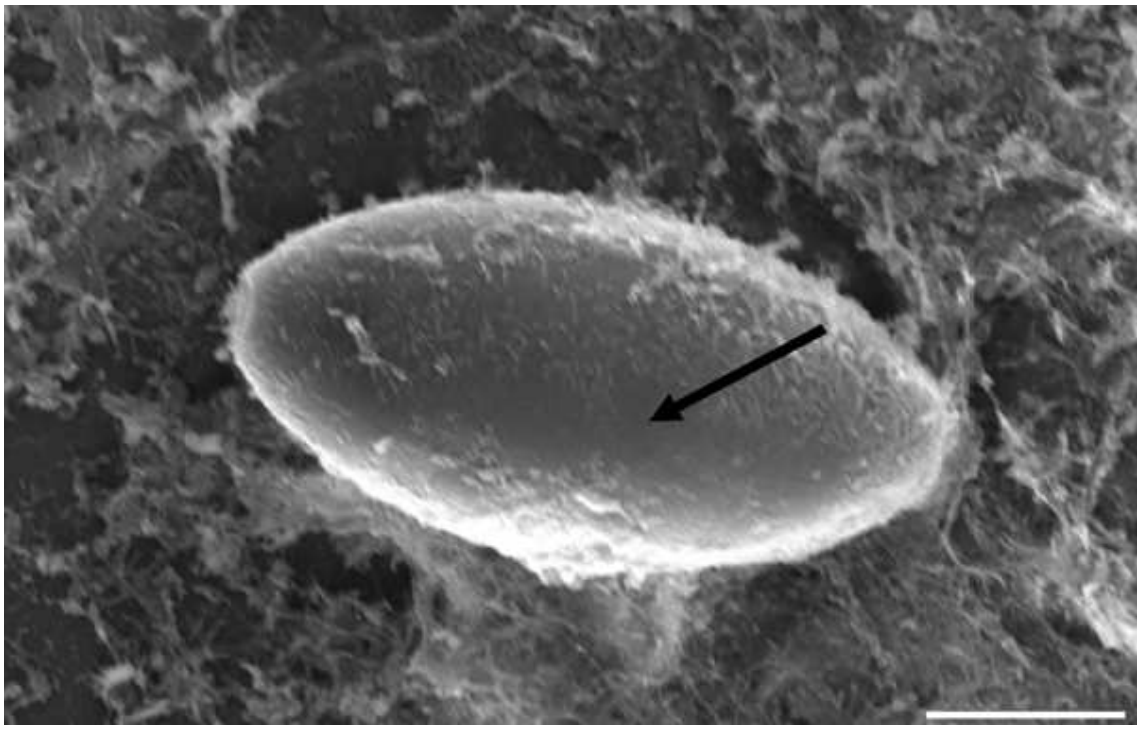

Fig. 4. *Trichuris trichiura* eggs (black arrow) without fungus (control). Bar: 20μm.

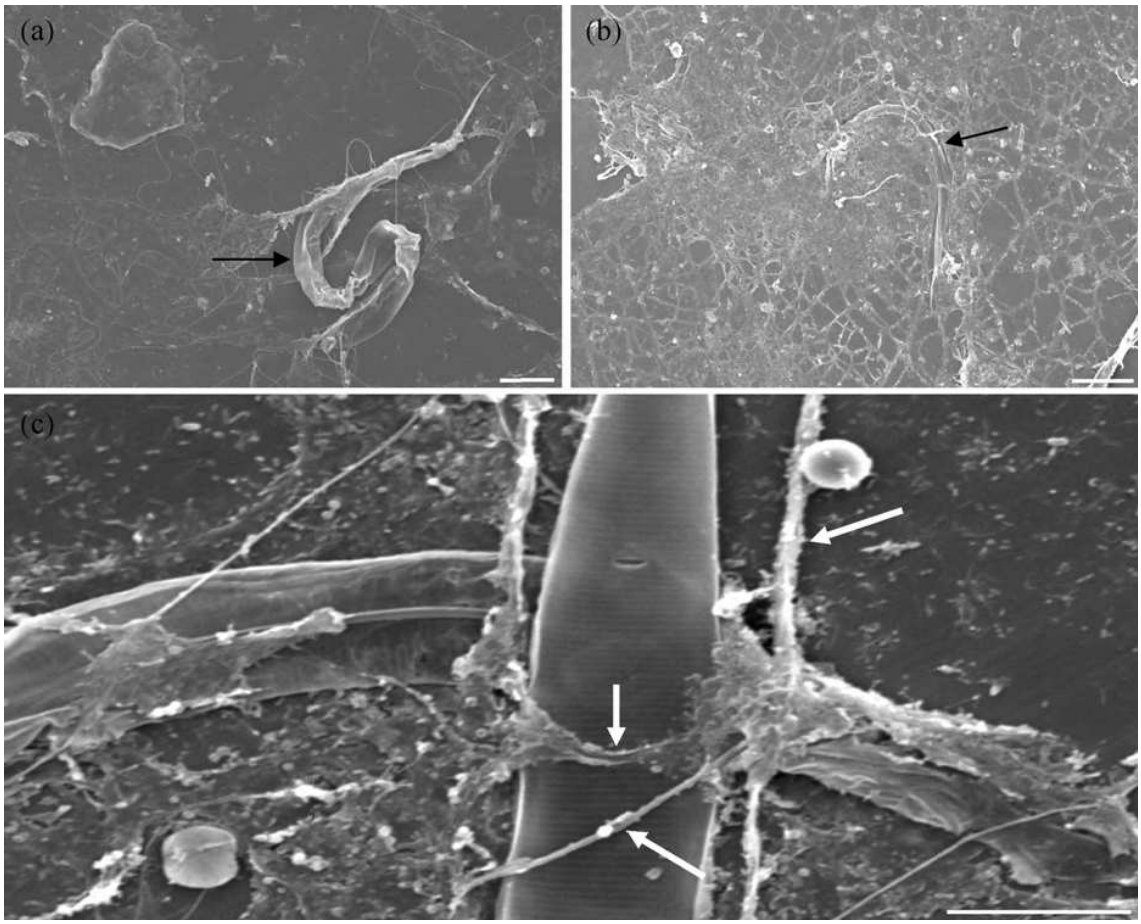

Fig. 5. (a and c) Infective larvae (L3) of preyed *Haemonchus contortus* (black arrow) by predator fungi *Duddingtonia flagrans* (AC001) (a and c) and *Monacrosporium thaumasium* (NF34a) (b) with production of traps (black arrow), causing its destruction, after 6h. Scanning electron microscopy (SEM). Bar: (a) 40 µm; (b) 100 µm; (c) 20 µm.

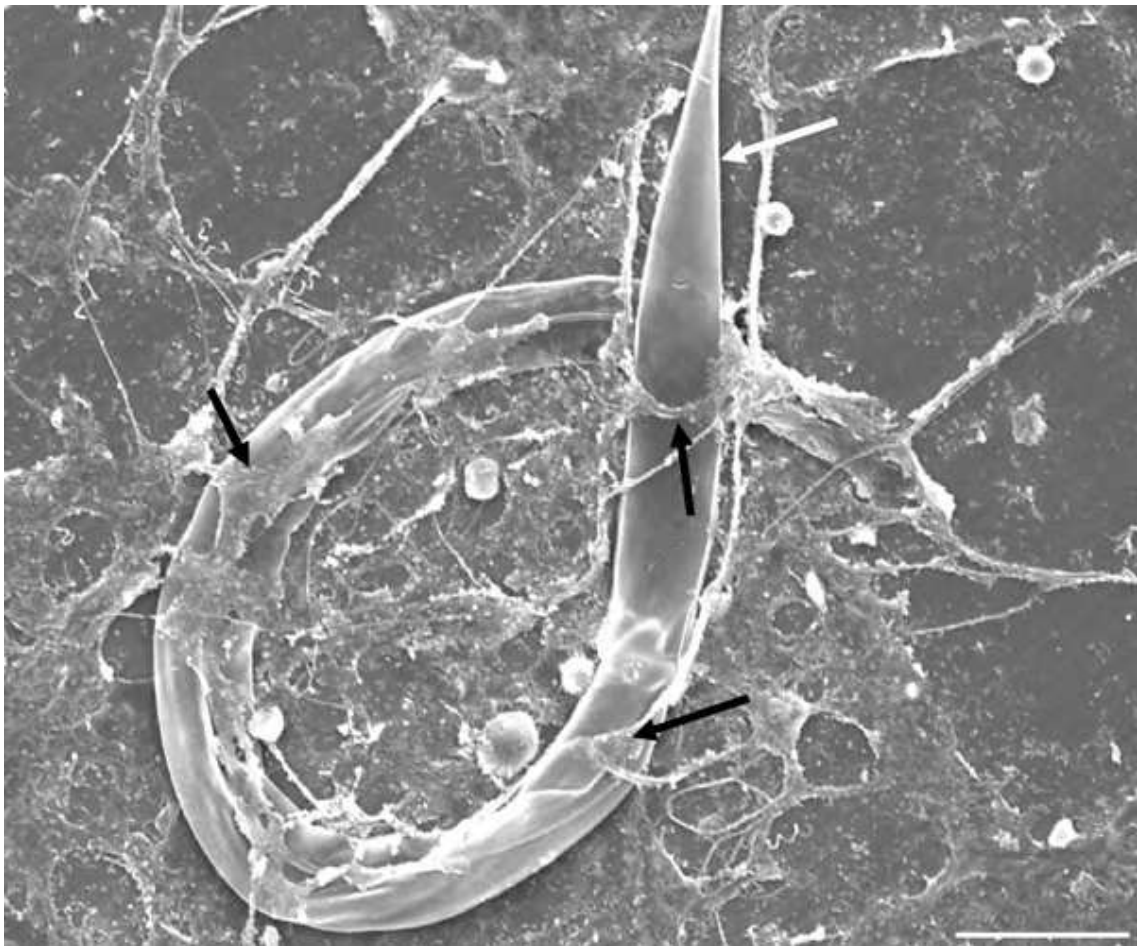

Fig. 6. SEM of infective larvae (L3) of preyed *Haemonchus contortus* (white arrow) by predator nematophagous fungi stimulating the production of traps (black arrow). Bar: 40μm.

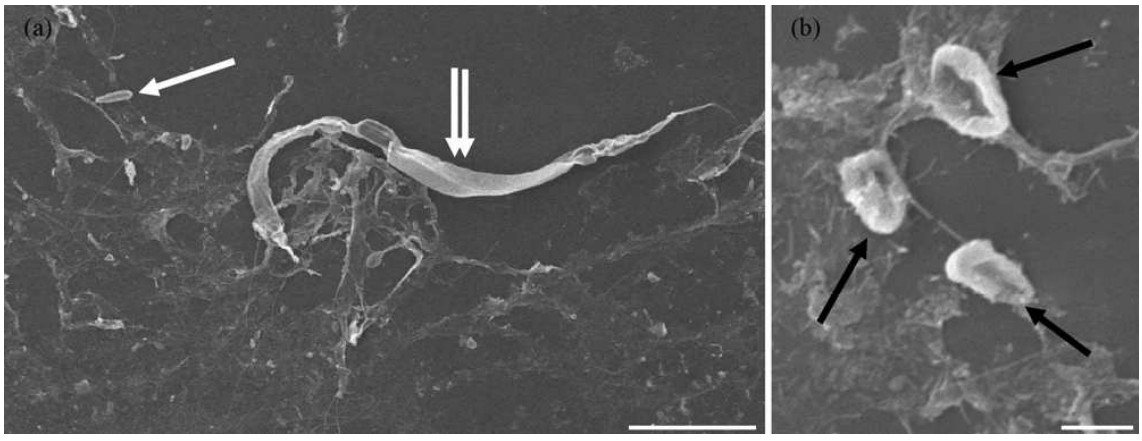

Fig. 7. (a and b) Observation of specific structures of the predator fungus *Duddingtonia flagrans* attached to infective larvae (L3) of *Haemonchus contortus* (white double arrow) and later causing its destruction. (a) Conidia (white arrow); (b) traps (black arrow). SEM. Bar: (a) 100μm; (b) 10μm.

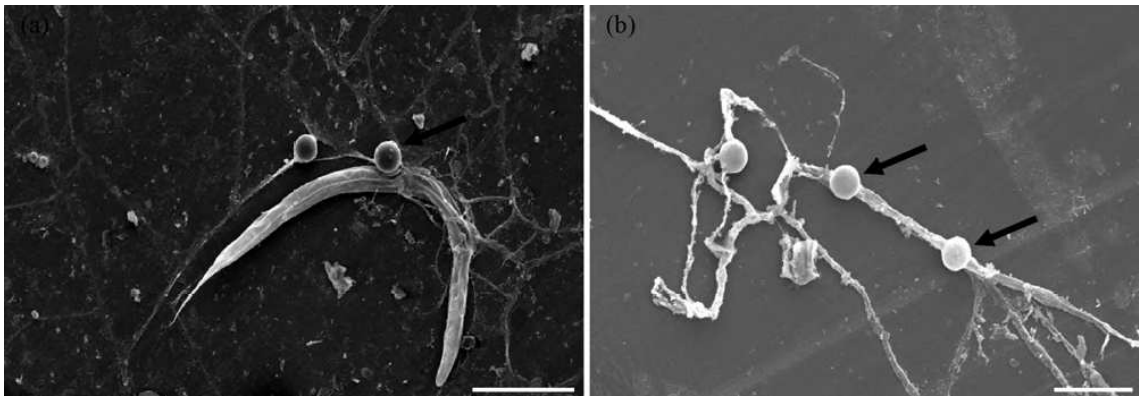

Fig. 8. (a and b) Production of chlamydospores (black arrow) of fungus *Duddingtonia flagrans* in Petri dishes. SEM. Bar: (a) 100µm; (b) 50µm.

(ii) Capítulo 4

In vitro ovicidal activity of the nematophagous fungi _Duddingtonia flagrans_, _Monacrosporium thaumasium_ and _Pochonia chlamydosporia_ on _Trichuris vulpis eggs_

Veterinary Parasitology, v.172, p. 76-79, 2010.
DOI: 10. 1016/j.vetpar. 2010.04.034

Abstract

Nematophagous fungi are a viable and promising alternative. The *in vitro* effect of four isolates of the nematophagous fungi *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34a) and *Pochonia chlamydosporia* (VC1 and VC4) on the eggs of *Trichuris vulpis* was evaluated. One thousand eggs of *T. vulpis* were plated on Petri dishes with 2% water-agar with the fungal isolates grown and without fungus as control. After seven, 14 and 21 days a hundred eggs were removed from each plate and classified according to the following parameters: type 1, lytic effect without morphological damage to eggshell; type 2, lytic effect with morphological alteration of embryo and eggshell; and type 3, lytic effect with morphological alteration of embryo and eggshell, besides hyphal penetration and internal egg colonization. *P. chlamydosporia* demonstrated ovicidal activity ($p < 0.05$) on the eggs of *T. vulpis* in the studied intervals presenting type 3 effect of 29.5% (VC1) and 36.5% (VC4), 59.5% (VC1) and 2.5% (VC4), 94.8% (VC1) and 2.95% (VC4) at seven, 14 and 21 days, respectively. The other fungi showed no type 3 effect. *P. chlamydosporia* should be a potential biological control agent of *T. vulpis* eggs.

Keywords: Nematophagous fungi; *Duddingtonia flagrans, Monacrosporium thaumasium*; *Pochonia chlamydosporia; Trichuris vulpis*; Biological control.

Introduction

The gastrointestinal parasite nematode *Trichuris vulpis* is worldwide distributed and commonly found in dogs causing many unwanted reactions in the host, such as alterations of the immunological response, decrease in the nutritional conversion, and predisposition to hosting various pathogenic microorganisms. The parasite's eggs are eliminated in the environment with the host's feces, surviving for several years and being a source of infection for other canids and even for human, in which they will fulfill their biological cycle (Urquhart et al. 1998; Schimmel et al., 2009).

The slow development of *T. vulpis*, together with the subclinical signs, could be the reason why there is a general belief that the *T. vulpis* infections in dogs may be less pathogenic. However, the infections can reduce growth in puppies due to the resistance to some anthelminthic drugs. In this way, alternative control measures for domestic animals' parasitoses have been the target of many researchers around the world. Among these, nematophagous fungi stand out as a viable and promising control alternative (Braga et al., 2008a, 2010; Silva et al., 2009).

Nematophagous fungi are classified into predators, endoparasites, and opportunists. These are cosmopolitan fungi, occurring in natural and agricultural soils and in all types of organic matter in decomposition (Araújo et al., 2004). In the group of predators fungi, the genera *Arthrobotrys*, *Duddingtonia*, and *Monacrosporium* stand out for their effective environmental control of nematodes by forming traps (Dimander et al., 2003; Silva et al., 2009). Among the opportunistic fungi, the species *Pochonia chlamydosporia* stands out (Gams and Zaire, 2001). According to Lysek and Sterba (1991), the action of this fungus is based on appressorial formation, developed from undifferentiated hyphae, which allows the colonization of the egg surface and penetration through both mechanical and enzymatic actions, characterizing a type 3 effect (eggs destruction).

The objective of the present study was to evaluate the *in vitro* action of the nematophagous fungi *Duddingtonia flagrans*, *Monacrosporium thaumasium,* and *P. chlamydosporia* on *T. vulpis* eggs.

Material and methods

Fungi

Four isolates of nematophagous fungi: one from de *D. flagrans* (AC001), one from *M. thaumasium* (NF34a), and two from *P. chlamydosporia* (VC1 and VC4) were kept in test tubes containing 2% corn–meal–agar, in the dark, at 4° C for 10 days. Culture disks, 4 mm in diameter, were extracted from fungi isolates kept in the test tubes and plated into 9 cm diameter Petri dishes containing 20 mL of 2% potato–dextrose–agar, and then stored in the dark, at 25° C for 10 days. After growth of the isolates, new culture disks, 4 mm in diameter, were transferred to 9 cm diameter Petri dishes containing 20 mL of 2% water–agar culture medium (2% WA) and for 10 days.

Obtaining of *T. vulpis* eggs

Eggs of *T. vulpis* were recovered from the dissection of an adult female specimen obtained from the large intestines of dogs naturally infected, dead from natural causes, and further necropsied at the Veterinary Department of the Universidade Federal de Viçosa. The eggs were recovered from an adult worm. The identification of the parasite should be enough to attribute a species to the eggs. Eggs were identified according to the parameters set by Urquhart et al. (1998).

Experimental assay

Eggs were morphologically analyzed for their integrity under light microscopy (10X objectives) and plated on 9.0 cm diameter Petri dishes with 2% WA culture medium with the fungal isolates grown for 10 days and control without fungus, with 10 repetitions per group. Each plate containing one thousand *T. vulpis* eggs was assayed against one fungal isolate only. At the intervals seven, 14 and 21 days, approximately 100 eggs were removed from each plate according to Araújo et al. (1995), placed in glass slides with a drop of 1% Amam blue corant and examined under a 40X lens according to Lysek et al. (1982): type 1, lytic effect without morphological damage to eggshell, with hyphae adhered to the shell; type 2, lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration

through the eggshell; and type 3, lytic effect with morphological alteration of embryo and eggshell, with hyphal penetration and internal colonization.

Statistical analysis

Data from each studied interval were examined by nonparametric Friedman test at 5% probability (Ayres et al., 2003).

Results

The ovicidal activity of the fungi is shown in Table 1. Data analysis showed difference ($P < 0.05$) for ovicidal activity among the studied isolates, demonstrating that both VC1 and VC4 isolates of *P. chlamydosporia* showed type 3 effect of 29.5% and 36.5%; 59.5% and 2.5%; 94.8% and 2.9%, for the intervals seven, 14 and 21 (Figure 1) days respectively. The percentages found characterize *P. chlamydosporia* as an ovicidal fungus; that VC1 has a strong ovicidal activity between 14 and 21 days. However, there were no larger variations in the activity among the days of observation, reaching maximum ovicidal activity between 14 and 21 days. *T. vulpis* eggs destruction was not observed in the control group during the experimental assay (Figure 2).

The fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34a) only had type 1 effect on *T. vulpis* eggs, not showing types 2 and 3 effects, with no injury or destruction of the eggs.

Discussion

The fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34a) did not show ovicidal activity, since Lysek (1976) only considers as ovicidal the fungi with type 3 effect on nematode eggs. In a study with trematode eggs, Braga et al. (2008a, b) registered that these fungi also showed only type 1 effect on the eggs of *Fasciola hepatica* and *Schistosoma mansoni* in the intervals seven, 14 and 21 days. Although *D. flagrans* and *M. thaumasium* have been recognized as biocontrol agents of nematode larvae, in this work, hyphae of both fungi that were adhered to the eggshell did not cause egg destruction. A viability test should comprehend the administration of treated eggs to

susceptible animals that have been previously in contact with the fungi (Morgan-Jones et al., 1983).

The first mode of mechanical action mentioned for an ovicidal fungus is the appressorium, which is the structure used for egg penetration (Lysek, 1978). Besides, according to Stirling and West (1991), the direct effect of fungal parasitism on embryo development is through the enzymatic action on the eggshell by increasing permeability and facilitating the passage of toxins.

In the present work, these isolates had greater percentages of ovicidal activity on *T. vulpis* eggs compared with the reports by Braga et al. (2007) with eggs of *A. lumbricoides*. The results obtained with *A. lumbricoides* with the isolates VC1 and VC4 were more homogeneous, even if the ovicidal activity was lower compared with *D. flagrans* and *M. sinense* in the intervals seven, 10 and 14 days of interaction, observing that isolates VC1 and VC4 showed type 3 effect of 20% and 18%; 25% and 22%; and 30% and 26%, respectively. These results indicate there are differences in the interaction process between the isolates and the eggs of the studied nematodes. In this way, further research is necessary for greater elucidation of this fact. *P. chlamydosporia* has been successfully tested on eggs of various genera of gastrointestinal parasite helminths (Araújo et al., 2008; Braga et al., 2007; Braga et al., 2010). However, the present work is the first report on ovicidal activity on *T. vulpis* eggs.

Conclusion

Pochonia chlamydosporia was effective in destroying *in vitro T. vulpis* eggs and may contribute to decrease environmental contamination by eggs this nematode.

Acknowledgments

This study was supported by the Research Project of the Universidade Federal de Viçosa, Brazil. Thanks are also due to FAPEMIG for the financial support and CAPES for the scholarships granted.

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Table 1 - Percentages and standard deviation of ovicidal activity for the nematophagous fungus *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34a), *Pochonia chlamydosporia* (VC1 and VC4) and the control without fungal treatment, against eggs of *Trichuris vulpis* after seven,14 and 21 days of interaction.

Seven Days of interaction				
Isolates	Without alteration+	Effect type 1*	Effect type 2**	Effect type 3***
AC001	51 ^{A} ± 7.38	49 ^{A} ± 7.38	0 ^{A} ± 0	0 ^{A} ± 0
NF34a	47.5 ^{A} ± 11.36	52.5 ^{A} ± 11.36	0 ^{A} ± 0	0 ^{A} ± 0
VC1	5 ^{B} ± 6.24	23 ^{B} ± 8.57	42.5 ^{B} ± 10.07	29.5 ^{B} ± 9.27
VC4	9.5 ^{B} ± 4.38	18.5 ^{B} ± 6.69	36 ^{B} ± 9.37	36.5 ^{B} ± 36.5
Control	100 ^{C} ± 0	0 ^{C} ± 0	0 ^{C} ± 0	0 ^{C} ± 0
14 Days of interaction				
Isolates	Without alteration+	Effect type 1*	Effect type 2**	Effect type 3***
AC001	42^{A} ± 12.06	57 ^{A} ± 10.85	0 ^{A} ± 0	0 ^{A} ± 0
NF34a	1.2 ^{AB}± 1.03	98.7 ^{AB} ± 0.95	0 ^{A} ± 0	0 ^{A} ± 0
VC1	0.5 ^{B}± 1.58	55 ^{A} ± 8.64	35.5 ^{B} ± 16.74	59.5 ^{B} ± 22.79
VC4	2 ^{B}± 6.33	5.5 ^{A} ± 9.56	19.5 ^{B} ± 9.85	2.5 ^{B} ± 73
Control	100 ^{C} ± 0	0 ^{C} ± 0	0 ^{C} ± 0	0 ^{C} ± 0
21 Days of interaction				
Isolates	Without alteration+	Effect type 1*	Effect type 2**	Effect type 3***
AC001	33 ^{A} ± 5.87	67 ^{A} ± 5.87	0 ^{A} ± 0	0 ^{A} ± 0
NF34a	1.1 ^{A} ± 0.74	98.9 ^{A} ± 0.74	0 ^{A} ± 0	0 ^{A} ± 0
VC1	0 ^{B} ± 0	0.8 ^{AB} ± 1.62	4.4 ^{B} ±3.13	94.8 ^{B} ± 4.1
VC4	0 ^{B} ± 6.68	0.2 ^{B} ± 10.42	4.1 ^{B} ± 5.8	2.95 ^{B} ± 95.7
Control	100 ^{C} ± 0	0 ^{C} ± 0	0 ^{C} ± 0	0 ± 0

Percentages followed by same letter (A, B, C) in the same column are not significantly different ($p > 0.05$) – Friedman test. + Without alteration. *Effect type 1, lytic effect without morphological damage to eggshell, with hyphae adhered to the shell. **Effect type 2, lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell. ***Effect type 3, lytic effect with morphological alteration of embryo and eggshell, with hyphal penetration and internal colonization.

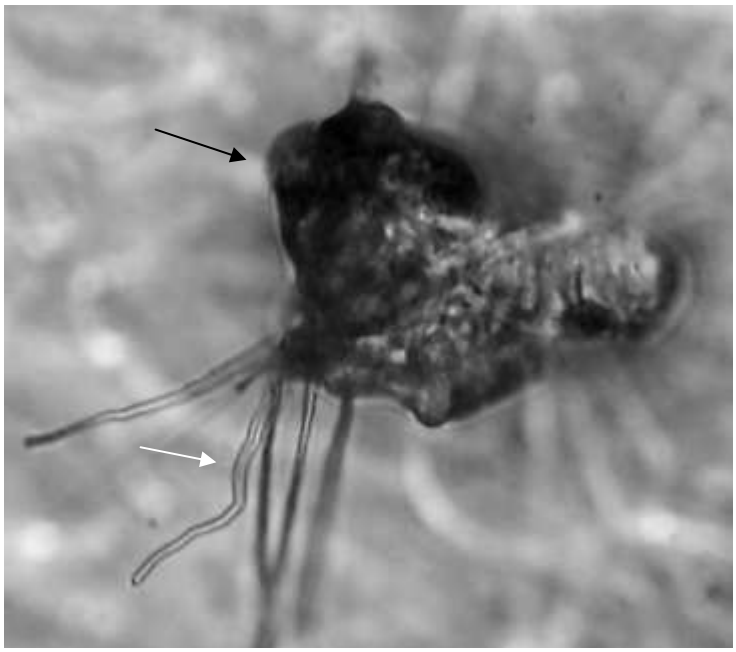

Figure 1 - Broken eggs of *Trichuris vulpis* (white arrow) and hyphae the *Pochonia chlamydosporia* (black arrow), after 21 days of interaction. Optical Microscopy - 40x objective lens.

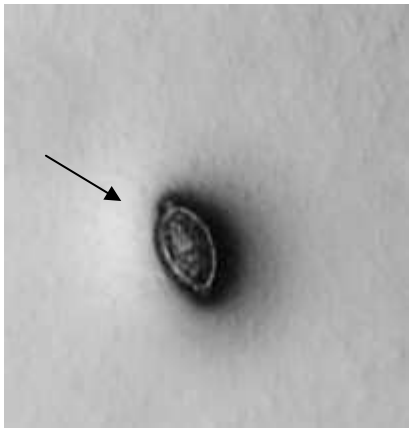

Figure 2 – Show *Trichuris vulpis* of the control group, without fungus.

(ii) Capítulo 5

Destruction of *Anoplocephala perfoliata* Eggs by the Nematophagous Fungus *Pochonia chlamydosporia*

Journal of Equine Veterinary Science, v.30, p. 701-704, 2010.
DOI: 10.1016/j.jevs.2010.11.004
Available online 14 December 2010.

ABSTRACT

Nematophagous fungi are a viable and promising alternative in the biological control of helminths. The *in vitro* effect of an isolate of the nematophagous fungus *Pochonia chlamydosporia* (VC1) on eggs of *Anoplocephala perfoliata* was evaluated. The eggs were morphologically analyzed for their integrity under light microscopy (10x objectives), plated on 9.0 cm diameter petri dishes containing 2% WA culture medium with and without fungal isolate (control), grown for 10 days, and 10 replicates were prepared per group. In all, 1000 eggs of *A. perfoliata* were plated on petri dishes containing 2% water agar culture medium with (VC1) and without the fungal isolate (control). After three, five, seven and 10 days, approximately 100 eggs were removed from each plate and classified on the basis of the following parameters: without alteration; type 1, lytic effect without morphological damage to eggshell; type 2, lytic effect with morphological alteration of embryo and eggshell; and type 3, lytic effect with morphological alteration of embryo and eggshell, in addition to hyphal penetration and internal egg colonization and destruction. The *P. chlamydosporia* fungus demonstrated ovicidal activity ($P < 0.05$) on the eggs of *A. perfoliata* in the studied intervals presenting type 3 effect of 35%, 42.5%, 53.83% and 71.17% for the intervals three, five, seven and 10 days, respectively. *P. chlamydosporia* is a potential biological control agent for the eggs of *A. perfoliata*.

Keywords: Nematophagous fungus; *Pochonia chlamydosporia; Anoplocephala perfoliata*; Biological control

INTRODUCTION

Equids are natural hosts of a great number of helminths. They present a vast parasite fauna, including various helminths families with different genera, which may cause signs ranging from mild abdominal discomfort to fulminant colic episodes and death.[1] Tapeworms are no longer considered non-pathogenic parasites but are now acknowledged as potentially harmful. Damage is specifically related to the location of one of the species.[2] The cestode *A. perfoliata,* a large intestine parasite that causes colic episodes, intussusception in the ileocaecal junction, and has a tendency to locate in the same junction, may lead to the death of the animal that it infects.[3]

Control measures adopted by most horse breeding farms are exclusively based on the use of antiparasitic drugs for their practicality and efficiency. The availability of alternate classes of cestocides represents an opportunity to avoid or minimize selection for resistance.[1,4,5] Many researchers around the world are targeting alternative control measures for this and other types of gastrointestinal helminthiases because resistance to the chemical groups is widespread. Among these alternatives is the biological control with the use of nematophagous fungi.[6] Nematophagous fungi are a viable and promising alternative. They can be divided in three groups: predators, endoparasites, and opportunists (with ovicidal activity). Among the opportunist fungi, the species *Pochonia chlamydosporia* has been successfully used under laboratory conditions and in the field to control the eggs of gastrointestinal parasitic helminths present in many domestic animals, including equines.[7,8] However, this fungus has not yet been tested on equine tapeworm eggs.

The objective of this work was to evaluate *in vitro* ovicidal action of the nematophagous fungus *Pochonia chlamydosporia* on *Anoplocephala perfoliata* eggs.

MATERIAL AND METHODS

Fungus

One isolate of the fungus *P. chlamydosporia* (VC1) was maintained at 4 ºC, protected from light, in assay tubes containing corn–meal–agar 2%. This isolate was previously stored at the Laboratory of Parasitology of the

Veterinary Department of the Universidade Federal de Viçosa. Culture samples were extracted from that isolate and plated on 9.0-cm diameter petri dishes containing 20 ml of 2% potato dextrose agar, the plate were then kept at 25 ºC in the dark for 10 days.

Obtaining of *A. perfoliata* eggs

Eggs of *A. perfoliata* were removed from the proglottids of an adult specimen obtained from the large intestines of a parasitized horse, dead from gastrointestinal complications (intussusception, Fig. 1) at the Large Animals Hospital of the Veterinary Department of the Universidade Federal de Viçosa. The eggs were analyzed under light microscopy (10x objectives)[9] and were washed 10 times in distilled water and centrifuged at 1000 rpm for 5 min each time. The supernatant was discarded at the end of each centrifugation cycle. The eggs were incubated at 25º C for 14 days with a solution containing 0.05% formalin, 0.005% streptomycin sulphate and 0.01% chloramphenicol.[10]

Experimental assay

The morphological integrity of the eggs was analyzed under light microscopy (10x objectives). They were transferred to 9.0 cm diameter petri dishes containing 2% WA culture medium with the fungal isolate that grew for 10 days and without fungus (control), with 10 repetitions per group. Each plate contained 1,000 *A. perfoliata* eggs that were assayed against one fungal isolate only and control plates (without fungus) also contained 1,000 eggs. At three, five, seven and 10 days of culture, approximately 100 eggs were removed from each plate,[10] placed on glass slides with a drop of 1% Amam blue corant and examined under a 40X lens,[11] and classified into the following groups: Without alteration; type 1, lytic effect without morphological damage to eggshell, with hyphae adhered to the shell; type 2, lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell; and type 3, lytic effect with morphological alteration of embryo and eggshell, with hyphal penetration and internal colonization and destruction.

Statistical analysis

Data for each interval (three, five, seven and 10 days) and treatment (treated vs controls) were analyzed by Friedman nonparametric test with 5% probability.[12]

RESULTS

On the basis of statistical analysis, there were differences ($P < 0.05$) with respect to ovicidal activity among the isolate groups, demonstrating that the VC1 isolate of *P. chlamydosporia* showed type 3 effect of 35%, 42.5%, 53.8%, 71.2% for the intervals of three, five, seven and 10 days respectively (Table 1). However, there were no greater variations in the activity with respect to the intervals of observation, reaching maximum ovicidal activity between seven and 10 days. *A. perfoliata* eggs destruction was not observed in the control group during the experimental assay (Fig. 2 B). In general, the mean percent of eggs in type 1 and type 2 (Fig. 3) groups decreased over time, whereas the mean percent of eggs in type 3 increased (Fig. 2 A).

DISCUSSION

The high prevalence (100%) of *A. perfoliata*, even in the older ones, is not unusual in a herd of horses. For this to occur, the correct species of oribatid intermediate host mites plus in tapeworm eggs are requiered to be present in large numbers.[13]

Fungal egg parasitism is an important biological phenomenon that may be used as an alternative method of control of helminthiasis in domestic animals. The fungi *P. chlamydosporia* (VC1) has been shown to have ovicidal activity because it demonstrated type 3 effect on nematode eggs. [14] The first mode of mechanical action that has been mentioned for an ovicidal fungus is the appressorium, which is the structure used for egg penetration.[15] The direct effect of fungal parasitism on embryo development[16] is through the enzymatic action on the eggshell by increasing permeability and facilitating the passage of toxins. Sixty-four fungal isolates, including *P. chlamydosporia*, on eggs of *Meloidogyne javanica*[17] was analyzed and it was found that *P. chlamydosporia* had the highest ovicidal activity, similar to the results found in this study. Using a different *P. chlamydosporia* isolate (VC1) against *T.*

taeniaeformis eggs, Braga et al[18] reported type 3 effect of 32.2% for at seven days. In this study, the isolate VC1 showed type 3 effect of 53.83% at the same interval. Comparing the results obtained in this study for the type 3 effect, it was found that although the isolate VC1 showed higher activity against the eggs after seven days of interaction, overall, the ovicidal percentages were very similar. A greater action of *P. chlamydosporia* on eggs of *Taenia taeniaeformis* was found at in the intervals seven, 14, and 21 days of interaction Braga et al (2010). *P. chlamydosporia* (VC1) had greater percentages of ovicidal activity on *A. perfoliata* eggs after 10 days. What indicate differences in the interaction process between the isolates and the eggs of the studied cestode. Further research is necessary to elucidate this fact. *P. chlamydosporia* has been successfully tested on eggs of various genera of gastrointestinal parasite helminthes.[8,19] But this is the first report of its ovicidal activity on *A. perfoliata* eggs.

CONCLUSION

Pochonia chlamydosporia was effective in destroying *in vitro A. perfoliata* eggs and may contribute to decrease environmental contamination by these cestode eggs.

ACKNOWLEDGMENTS

This study was supported by the Research Project of the Universidade Federal de Viçosa, Brazil. The authors thank FAPEMIG for the financial support and CAPES for the scholarships granted.

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Table 1 - Percentages and (±) standard deviation of ovicidal activity for the nematophagous fungus *Pochonia chlamydosporia* (VC1) and the control without fungal treatment, on *Anoplocephala perfoliata* eggs after three, five, seven and 10 days of interaction

Three days of interaction				
Groups	Without alteration	Effect type 1	Effect type 2	Effect type 3
VC1	$5^{A} \pm 3.16$	$21.67^{A} \pm 5.16$	$38.33^{A} \pm 6.05$	$35^{A} \pm 7.07$
Control	$100^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$
Five days of interaction				
Groups	Without alteration	Effect type 1	Effect type 2	Effect type 3
VC1	$5^{A} \pm 6.32$	$17.5^{A} \pm 6.89$	$30.0^{A} \pm 7.75$	$42.5^{A} \pm 7.58$
Control	$100^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$
Seven days of interaction				
Groups	Without alteration	Effect type 1	Effect type 2	Effect type 3
VC1	$2.83^{A} \pm 3.19$	$13^{A} \pm 4$	$30.3^{A} \pm 5.71$	$53.83^{A} \pm 11.05$
Control	$100^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$
10 days of interaction				
Groups	Without alteration	Effect type 1	Effect type 2	Effect type 3
VC1	$0^{A} \pm 0$	$6.67^{A} \pm 2.87$	$22.17^{A} \pm 4.49$	$71.17^{A} \pm 4.17$
Control	$100^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$	$0^{B} \pm 0$

Percentages followed by same letter (A and B) in the same column are not significantly different ($p>0.05$) – Friedman test. + Without alteration. *Effect type 1, lytic effect without morphological damage to eggshell, with hyphae adhered to the shell. **Effect type 2, lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell. ***Effect type 3, lytic effect with morphological alteration of embryo and eggshell, with hyphal penetration and internal colonization and destruction.

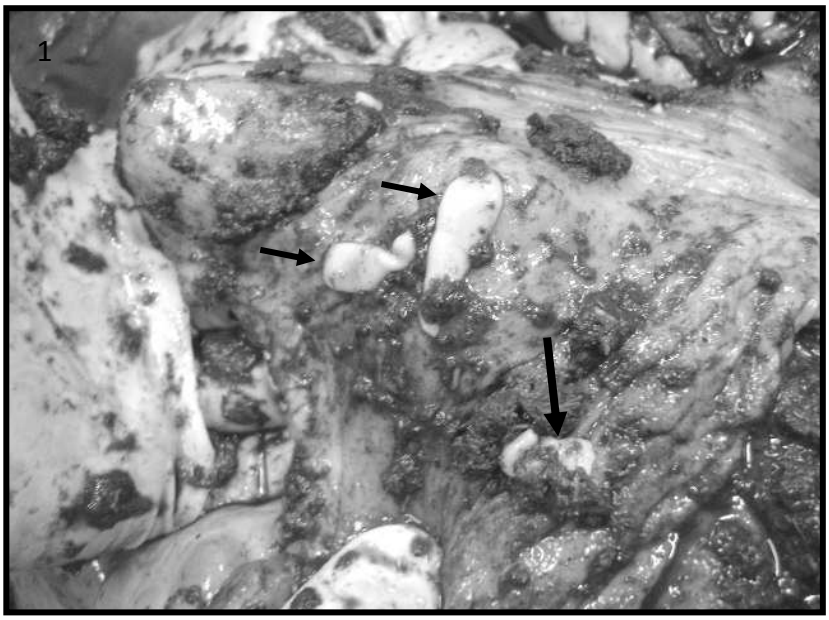

Figure 1 – Proglottids (black arrow) of an adult specimen (*Anoplocephala perfoliata*) obtained from the large intestines of parasitized horse, dead from gastrointestinal complications (intussusception).

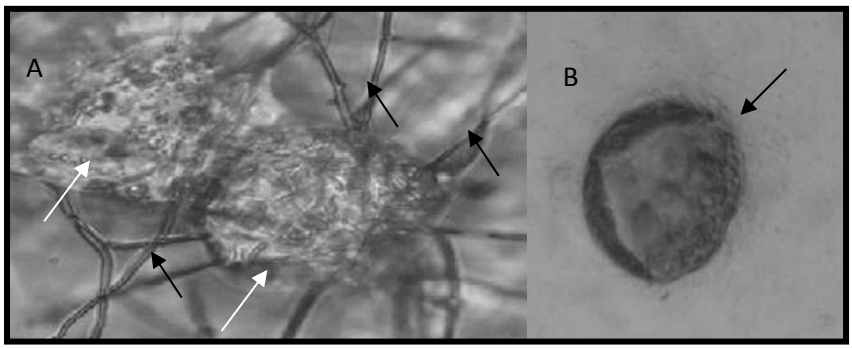

Figure 2 (A and B) – (A) Broken eggs of *Anoplocephala perfoliata* (white arrow) and hyphae the *Pochonia chlamydosporia* (black arrow), after 10 days of interaction (effect type 3). Optical microscopy – (40x objective). **(B)** *A. perfoliata* egg (black arrow) of the control group, without fungus.

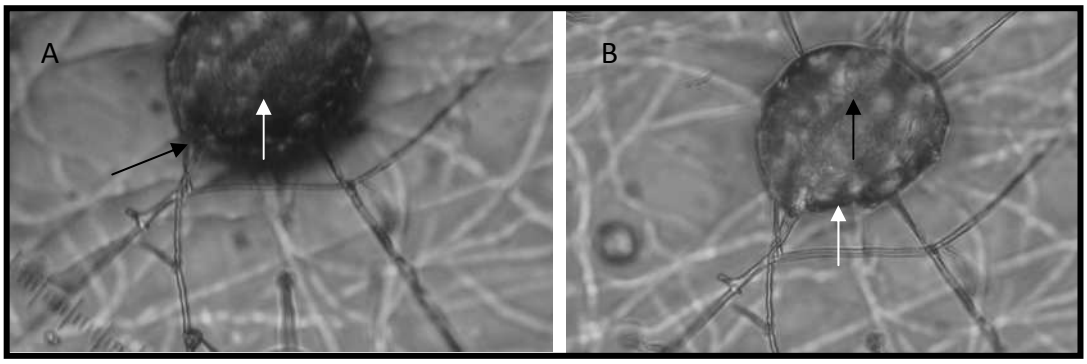

Figure 3 (A) *Anoplocephala perfoliata* eggs (white arrow) and hyphae the *Pochonia chlamydosporia* (black arrow), effect type 1. Optical microscopy (40x objective). **(B)** *A. perfoliata* egg (white arrow), lytic effect with morphological alteration of embryo and eggshell (effect type 2).

(iii) Conclusões Gerais

I. O tratamento de ovinos com péletes contendo os fungos nematófagos *Duddingtonia flagrans* (AC001) *e Monacrosporium thaumasium* (NF34a) pode ser utilizado como uma alternativa no controle das nematodioses gastrintestinais de ovinos.

II. Os isolados fúngicos *D. flagrans* (AC001) e *M. thaumasium* (NF34a) foram efetivos no controle *in vitro* de larvas infectantes (L3) de *Haemonchus contortus* de ovinos e podem ser usados no controle biológico deste nematóide.

III. A microscopia eletrônica de varredura demonstrou que o fungo *Pochonia chlamydosporia* (isolados VC1 e VC4) destruiu os ovos de *Trichuris trichiura* de humanos, mostrando pela primerira vez esta atividade ovicida. *D. flagrans* (AC001) e *M. thaumasium* (NF34a) destruíram L3 de *H. contortus* de ovinos. Os isolados de *P. chlamydosporia* (VC1 e VC4) podem ser controladores biológicos potenciais de *T. trichiura* de humanos e os fungos *D. flagrans* (AC001) e *M. thaumasium* (NF34a) de *H. contortus* de ovinos.

IV. *P. chlamydosporia* (VC1 e VC4) foi efetivo em destruir *in vitro* ovos de *Trichuris vulpis* de cães e podem contribuir para diminuir a contaminação ambiental por ovos deste nematóide.

V. *P. chlamydosporia* (VC1) foi efetivo em destruir *in vitro* ovos de *Anoplocephala perfoliata* e pode diminuir a contaminação ambiental por ovos deste cestoda.