FÁBIO RIBEIRO BRAGA

CONTROLE BIOLÓGICO DE NEMATODIOSES INTESTINAIS DE EQUINOS 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 Scientiae.*

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APROVADA: 28 de fevereiro de 2011.

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(Co-orientador)

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"A persistência é o caminho para o êxito"

Charles Chaplin

A Deus.
Ao meu filho, Davi.
A minha esposa, Gracilene.
Aos meus pais, Ricardo e Ângela.
Aos meus irmãos e amigos.

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BIOGRAFIA

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SUMÁRIO

LISTA DE TABELAS

Página

Página

CAPÍTULO 6

CAPÍTULO 7

Página

LISTA DE FIGURAS

Página

CAPÍTULO 1

CAPÍTULO 4

CAPÍTULO 5

Página

CAPÍTULO 6

CAPÍTULO 7

RESUMO

BRAGA, Fábio Ribeiro, D.Sc., Universidade Federal de Viçosa, fevereiro de 2011. **Controle biológico de nematodioses intestinais de equinos por fungos nematófagos.** Orientador: Jackson Victor de Araújo. Co-orientadores: José Humberto de Queiroz e Laércio dos Anjos Benjamin.

Com os objetivos de: testar uma formulação peletizada em matriz de alginato de sódio contendo massa miceliana do fungo *Duddingtonia flagrans* (isolado AC001) no controle biológico de ciatostomíneos de equinos criados a campo; avaliar a ação *in vitro* de quatro isolados de fungos nematófagos dos gêneros *D. flagrans* (isolado AC001), *Monacrosporium thaumasium* (isolado NF34a) e *Pochonia chlamydosporia* (isolados VC1 e VC4) sobre ovos de *Oxyuris equi* e *Austroxyuris finlaysoni;* avaliar a atividade ovicida *in vivo* do fungo *P. chlamydosporia* (isolado VC4) sobre ovos de *O. equi*; avaliar a capacidade predatória *in vitro* do fungo nematófago *D. flagrans* sobre larvas infectantes (L_3) de ciatostomíneos de equinos; testar a ação do extrato fúngico de *P. chlamydosporia* (VC4) sobre a eclosão de ovos de ciatostomíneos; otimizar a produção de protease pelo fungo *D. flagrans* (isolado AC001) e sua ação larvicida sobre ciatostomíneos de equinos e; avaliar a produção enzimática do extrato bruto e a atividade ovicida dos fungos *P. chlamydosporia* (VC1 e VC4) e *Paecilomyces lilacinus* (PL1) sobre ovos de *O. equi*, foram realizados ensaios experimentais em condições laboratoriais e a campo. Os animais do grupo tratado com péletes contendo o fungo *D. flagrans* (AC001) apresentaram diferença ($p<0,01$) na redução da contagem de ovos por grama de fezes e nas coproculturas quando comparados aos animais do grupo controle. Nos resultados dos ensaios experimentais *in vitro* observou-se que o fungo *P. chlamydosporia* (VC1 e VC4) influenciou de forma negativa os ovos de *O. equi* e *A. finlaysoni*. Foi observado também que o isolado de *P. chlamydosporia* (VC4) permaneceu viável e manteve sua atividade ovicida ($p<0,01$) após a passagem pelo aparelho gastrintestinal de equinos quando comparado ao grupo controle (sem fungo). Por outro lado, foi observada uma redução significativa ($p<0,01$) de 93,64% das L_3 de ciatostomíneos recuperadas das placas de Petri do grupo tratado com o fungo *D. flagrans* (AC001) em relação ao grupo controle. Contudo, em relação à ação do extrato fúngico de *P. chlamydosporia* (VC4) sobre a eclosão de ovos de ciatostomíneos, observou-se um percentual de redução na eclosão dos ovos de 72,8% (VC4) em relação ao grupo controle e redução ($p<0,01$) do número de L_3 de ciatostomíneos recuperadas das coproculturas (67,0%) do grupo tratado em relação ao controle, ao final dos ensaios. O sulfato de magnésio ($MgSO_4$), o sulfato de ($CuSO_4$) e a caseína otimizaram a

produção de proteases pelo fungo *D. flagrans* (AC001) em meio líquido demonstrando também que o extrato otimizado teve ação larvicida sobre ciatostomíneos. Com a produção enzimática do extrato bruto e sua atividade ovicida dos fungos *P. chlamydosporia* (VC1 e VC4) e *P. lilacinus* (PL1) sobre ovos de *O. equi*, observou-se que os fungos *P. chlamydosporia* (VC1 e VC4) e *P. lilacinus* (PL1) cresceram e produziram atividade enzimática nos meios ágar suplementados com gelatina (GA), caseína (CA), óleo de oliva (OOA) e amido (SSA). Além disso, ao final do experimento (15 dias) os isolados testados de VC1, VC4 e PL1 demonstraram atividade ovicida (efeito do tipo 3) de (77,6%; 54,0%; 51,8% e 67,8%), (72,4%; 50,0%; 58,4% e 77,6%) e 62,4%; 57,5%; 65,3% e 63,5%), respectivamente, nos meios GA, CA, OOA e SSA. Os resultados demonstrados sugerem que os fungos nematófagos *D. flagrans* (AC001), *Monacrosporium thaumasium* (NF34), *P. chlamydosporia* (VC1 e VC4) e *P. lilacinus* podem ser empregados no controle biológico de helmintos de equinos.

ABSTRACT

BRAGA, Fábio Ribeiro, D.Sc., Universidade Federal de Viçosa, February 2011. **Biological control of equine intestinal nematodiosis by nematophagous fungi.** Adviser: Jackson Victor de Araújo. Co-advisores: José Humberto de Queiroz and Laércio dos Anjos Benjamin.

With the aim of: testing a pellet formulation in sodium alginate containing *Duddingtonia flagrans* (isolate AC001) mycelial fungus for biological control cyathostomins of horses raised in field, evaluate *in vitro* effect of *D. flagrans* (isolate AC001), *Monacrosporium thaumasium* (isolate NF34a) and *P. chlamydosporia* (isolates VC1 and VC4) on *Oxyuris equi* and *Austroxyuris finlaysoni* eggs; evaluate the *in vivo* ovicidal activity of *P. chlamydosporia* (isolate VC4) fungus on *Oxyuris equi* eggs and to evaluate the predatory capacity *in vitro* of *D. flagrans* nematophagous fungus on horses cyathostomin infective larvae (L_3); test *P. chlamydosporia* (isolate VC4) fungal extract action on cyathostomins eggs eclosion; optimize the protease production by *D. flagrans* (isolate AC001) and its larvicidal action on horses cyathostomin and, to evaluate crude enzymatic extract production and ovicidal activity of *P. chlamydosporia* (isolates VC1 and VC4) and *Paecilomyces lilacinus* (PL1) on *O. equi* eggs, tests were conducted in experimental laboratory and in field conditions. The animals in the group treated with *D. flagrans* (AC001) pellets showed a different ($p<0.01$) reduction in egg count per gram of feces and in fecal cultures compared to control animals. *In vitro* laboratory tests results showed that *P. chlamydosporia* (VC1 and VC4) fungus influenced negatively *O. equi* and *A. finlaysoni* eggs. It was also observed that the isolate (VC4) remained viable and maintained its ovicidal activity ($p<0.01$) after passing through the gastrointestinal tract of horses when compared to control (without fungus). Moreover, we observed a significant reduction ($p<0.01$) of 93.64% in cyathostomin L_3 recovered from Petri dishes in the group treated with *D. flagrans* (AC001) comparing to the control group. However, in relation to *P. chlamydosporia* (VC4) fungal extract action on cyathostomins eggs eclosion, there was a percentage reduction in eggs eclosion of 72.8% (CV4) compared to control group and decreased ($p<0.01$) the number of cyathostomins L_3 recovered from stool cultures (67.0%) in the treated group compared with controls at the end of the experiment. Magnesium sulphate (MgSO4), copper sulfate (CuSO4) and casein optimized the protease production by *D. flagrans* (AC001) in liquid medium, also demonstrating that the extract has optimized larvicidal action. The crude enzymatic extract production and ovicidal activity of *P.*

chlamydosporia (VC1 and VC4) and *P. lilacinus* (PL1) on *O. equi* eggs, showed that *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) growth and produced enzyme activity in agar medium supplemented with gelatin (GA), casein (CA), olive oil (OOA) and starch (SSA). Moreover, at the end of the experiment (15 days) the isolates tested (VC1, VC4 and PL1) showed ovicidal activity (type 3 effect) of (77.6%, 54.0%, 51.8% and 67.8%), (72.4%, 50.0%, 58.4% and 77.6%) and (62.4%, 57.5%, 65.3% and 63.5%), respectively, in medium GA, CA, OOA and SSA. The results shown suggest that the nematophagous fungi *D. flagrans* (AC001), *Monacrosporium thaumasium* (NF34), *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* can be used in horse's helminths biological control.

1. INTRODUÇÃO GERAL

Criações de animais com fins produtivos, com destaque para os bovinos, ovinos, caprinos e equinos apresentam drásticas perdas econômicas associadas principalmente ao parasitismo por endoparasitos e ectoparasitos. No Brasil grande parte da criação ainda é feita em regime de pasto, o que leva as constantes infecções por parasitos presentes nas pastagens (Anualpec, 2003). Por outro lado, na criação comercial de animais silvestres em países com grandes diferenças regionais como o Brasil existem alguns entraves à produção comercial desses animais, e, dentre esses, podem-se mencionar as nematodioses gastrintestinais, que merecem destaque (Bonuti et al., 2002).

Nematóides gastrintestinais são comuns em equinos, representando um grupo de grande importância no Brasil, já que grande parte do rebanho encontra-se infectado. Além disso, esses animais apresentam uma grande variedade de parasitos em sua fauna helmíntica, e algumas espécies/gêneros são de relevada importância, como: *Parascaris equorum*, *Anoplocephala perfoliata*, *Oxyuris equi*, *Cyathostomum* spp., e *Strongylus* spp (Kaplan, 2002). *O. equi* relatado em cavalos no ano de 430 (DC) por Hipocrates é habitante comum principalmente do cólon menor desses animais, mas ocasionalmente pode ser encontrado também no cólon maior (Morgan & Hawkins, 1949). Segundo Urquhart et al. (1998) e Bowman et al. (2006), a maioria dos efeitos patogênicos desse parasito no intestino se deve aos hábitos alimentares das larvas infetantes de terceiro e de quarto estádios que resultam em pequenas erosões da mucosa. Contudo, um efeito mais importante é a irritação causada pelas fêmeas adultas durante a oviposição. Segundo Bowman et al. (2006) as fêmeas grávidas de *O. equi* migram para a parte inferior do cólon e reto e para fora do ânus para aderir massas de ovos pele do ânus e da região perianal. Essas massas de ovos consistem em um fluido pegajoso cinza-amarelado contendo de 8 mil a 60 mil ovos, causando intenso prurido anal.

Entre os nematóides parasitos gastrintestinais de animais silvestres está o *Austroxyuris finlaysoni*, um oxyurídeo de marsupiais (Bowman et al., 1996). Gomes et al. (2003) mencionam que, alguns desses marsupiais, como o gambá de orelha branca (*Didelphis albiventris*) possui importância médico-veterinária por ser reservatório de nematóides, fungos como o *Histoplasma capsulatum* e também do *Trypanosoma cruzi.* Além disso, pode atuar como hospedeiro definitivo ou reservatório de inúmeros parasitos, artrópodes (carrapatos e pulgas), que agem como ectoparasitos.

O controle das nematodioses gastrintestinais foi feito ao longo das décadas através do uso de antihelmínticos, muitas vezes de maneira indiscriminada e sem estratégias de controle adequadas, fato que conduziu a resistência destes parasitos a maior parte das classes de antihelmínticos disponíveis, principalmente aos benzimidazóis (Kaplan, 2002; Matthews et al., 2004). Nesse contexto, o controle biológico realizado com fungos nematófagos tem sido utilizado como uma alternativa de controle natural de formas infectantes (ovos e ou larvas) de nematóides, pois capturam e causam sua destruição. São divididos em três grupos: predadores, ovicidas e endoparasitas (Araújo et al., 2004). Braga et al. (2009a, b) mencionam que esses fungos nematófagos podem ser utilizados com sucesso no controle de nematóides parasitos gastrintestinais de animais domésticos e silvestres, e dentre esses, os pertencentes à subfamília Cyathostominae e o *O. equi*, que possuem grande prevalência em boa parte do território brasileiro.

No grupo dos predadores, destaca-se a espécie *Duddingtonia flagrans* considerada a mais promissora devido a sua grande produção de clamidósporos que são estruturas resistentes. Além disso, é classificada como predadora e produz uma série de enzimas, e dentre essas as proteases (Park et al., 2001; Meyer & Wiebe, 2003; Braga et al., 2010a). As espécies do gênero *Monacrosporium* são predadoras e caracterizadas por produzirem um único conídio em cada conidióforo e pela produção de redes adesivas, formando hifas septadas e ramificadas (Mota et al., 2003; Campos et al., 2008).

No grupo de fungos ovicidas destacam-se as espécies *Pochonia chlamydosporia* e *Paecilomyces lilacinus*. O fungo *P. chlamydosporia* produz enzimas extracelulares do tipo serino proteases que desenvolvem um papel importante na infecção e na destruição (atividade ovicida) dos ovos de geohelmintos (Segers et al., 1994; Braga et al., 2008a, b, 2009a; 2010b). *P. lilacinus* tem sido utilizado com sucesso em condições laboratoriais e a campo no controle biológico de ovos de vários gêneros de helmintos parasitos gastrintestinais (Araujo et al., 2010).

Proteases são as enzimas hidrolíticas de maior importância industrial, correspondendo a 60% do total das enzimas produzidas mundialmente (Rao et al., 1998). Além disso, as proteases de microorganismos, e dentre esses, de fungos nematófagos, são as mais significantes, sendo 40% do total de enzimas vendidas comercialmente (Godfrey and West, 1996; Meyer and Wiebe, 2003). Contudo, Rao et al. (1998) relatam que o custo de produção dessas enzimas ainda é um importante obstáculo para a sua aplicação na indústria. Sendo assim, sua produção ainda é limitada, devido aos custos dos substratos utilizados para o cultivo dos microorganismos. Portanto, a otimização da produção de proteases por microorganismos pode contribuir para a redução dos gastos de forma efetiva

(Joo and Chang, 2005). Por outro lado, as proteases produzidas por fungos nematófagos têm sido extensivamente estudadas, uma vez que tem relação na degradação da cutícula dos nematóides (Tunlid and Jansson, 1991). Gupta et al. (2002) mencionam que vários trabalhos têm objetivado a melhoria da produção de proteases por microorganismos por meio do uso de ferramentas estatísticas como o design Plackett–Burman e a metodologia de superfície de resposta (RSM). De acordo com Djekrif-Dakhmouche et al. (2006) o uso de ferramentas estatísticas como o design Plackett–Burman e a RSM têm sido utilizados para a otimização de meios de cultura. Por outro lado, segundo Hajji et al. (2008) tais ferramentas também são aplicadas para o entendimento das interações entre vários parâmetros usando um número mínimo de experimentos.

A metodologia de superfície de resposta é uma técnica que avalia as relações existentes entre um grupo de fatores experimentais controlados e os resultados observados de um critério selecionado (Ambati & Ayyanna, 2001). Isso inclui design fatorial e análise de regressão, que ajudam na validação dos fatores efetivos e na construção de blocos para o estudo das suas interações, servindo também para a seleção das condições ótimas das variáveis para uma resposta escolhida (Sharma et al., 2007).

Além disso, cada fungo tem a sua exigência específica em condições especiais o que causaria a maximização da produção dessas enzimas. Ainda, segundo Araújo et al. (2004) o desenvolvimento de formulações fúngicas para uso no controle biológico é um dos principais passos para a produção comercial destes microorganismos. Sendo assim, este fato poderia contribuir para pesquisas futuras que visem a produção industrial em larga escala de fungos nematófagos.

Contudo, nos últimos anos a sociedade tem priorizado aspectos ambientais, direcionando muitas pesquisas para a descoberta de novas substâncias bioativas que possam ser empregadas no manejo integrado de pragas e doenças, com menos efeitos negativos sobre o meio ambiente (Garcla et al., 2010). Dessa forma, a produção de novas metodologias e a aplicação de enzimas derivadas de fungos nematófagos são bem vindas.

2. OBJETIVOS

1. Testar uma formulação peletizada em matriz de alginato de sódio contendo massa miceliana do fungo *Duddingtonia flagrans* (isolado AC001) no controle biológico de ciatostomíneos de equinos criados a campo.

2. Avaliar a ação *in vitro* de fungos nematófagos das espécies *D. flagrans* (AC001), *Monacrosporium thaumasium* (NF34a) e *Pochonia chlamydosporia* (VC1 e VC4) sobre ovos de *Oxyuris equi* e *Austroxyuris finlaysoni* por meio de dois ensaios experimentais (A e B) nos intervalos de cinco, 10 e 15 dias de interação.

3. Avaliar *in vivo* o fungo nematófago *P. chlamydosporia* (VC4) quanto a sua capacidade de passagem pelo aparelho gastrintestinal de equinos, sua resistência e viabilidade, observando sua capacidade predatória sobre ovos de *O. equi.*

4. Avaliar *in vitro* a capacidade predatória do fungo *D. flagrans* (AC001) sobre larvas infectantes de ciatostomíneos.

5. Testar a ação do extrato do fungo *P. chlamydosporia* (VC4) sobre ovos de ciatostomíneos e em culturas de fezes em dois ensaios experimentais (A e B).

6. Otimizar a produção de protease do fungo *D. flagrans* (AC001) pelo método de superfície de resposta e avaliar sua ação larvicida sobre ciatostomíneos de equinos.

7. Avaliar o crescimento, a produção enzimática e o efeito ovicida dos fungos *P. chlamydosporia* e *Paecilomyces lilacinus* em meios ágar sólidos de cultura sobre ovos de *O. equi* e avaliar o potencial enzimático do extrato bruto de *P. chlamydosporia* (VC1 e VC4).

CAPÍTULO 1

Biological control of horse cyathostomin (Nematoda: Cyathostominae) with the nematophagous fungus *Duddingtonia flagrans* in tropical southeast Brazil

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Abstract

The viability of a fungal formulation using the nematode predator fungus *Duddingtonia flagrans* was evaluated for the biological control of horse cyathostomin. Two groups (fungus-treated and control), consisting of eight crossbred mares each, 3 to 18 years of age, were fed on *Cynodon* sp. pasture naturally infected with equine cyathostome larvae. Each animal of the treated group was orally administered sodium alginate mycelial pellets (1g/10 kg live weight/ week), during six months. Animals of the control group were not treated. Significant reduction ($p<0.01$) in the number of eggs per gram of feces and coprocultures was found for animals of the fungus treated group compared with the control group. There was difference ($p<0.01$) of 78.5% in pasture samples collected up to (0-20 cm) between the fungus treated group and the control group, during the experimental period (May to October). Difference of 82.5% ($p <0.01$) was found between the fungus treated group and the control group in the sampling distance (20-40 cm) from fecal pats. In the last three months of the experiment (August, September and October), animals treated with the fungus showed difference ($P <0.01$) for weight gain, 38 kg more than the control group. The treatment of horses with sodium alginate pellets containing the nematophagous fungus *D. flagrans* can be effective against cyathostomin in tropical southeastern Brazil.

Keywords: Nematophagous fungi; *Duddingtonia flagrans;* cyathostomin; horse; biological control.

1. Introduction

A large variety of helminths are known to parasite horses. Nematodes, mainly cyathostomin species, are the most common and important among them. Also known as small strongyles, cyathostomin infections are responsible for causing anemia, weight loss, intestinal colic, and death in horses (Assis and Araújo, 2003). They are the most prevalent parasites in horses, present throughout the year in the pasture, having however greatly varied distribution in different age groups (Barbosa et al., 2001; Quinelato et al., 2008).

Klei and Chapman (1999) reported field data suggesting that horses can acquire resistance to helminths with age, which is confirmed by the reduced parasite load and egg count in feces. This response is slow and inconsistent in most animals and unrelated to the intensity of previous contact with parasite.

Kaplan (2002) and Matthews et al. (2004) discussed that worm control in horses is usually carried out with antihelmintic drugs, which have not been totally effective for the control of these nematodes since their action is restricted to adult parasites and the occurrence of resistance.

However, the continued use of the same antihelmintic class, as well as the rapid rotation of compound groups, introduction of resistant worms and the use of doses lower than the recommendation should be avoided (Mota et al., 2003). Biological control using natural nematode antagonistic fungi is among the most viable alternatives. These organisms comprise different types of fungi divided into predators, endoparasites and opportunists, whose action is concentrated in the fecal environment and directed against free-living parasitic larvae. In the group of predators, the species *Duddingtonia flagrans* stands out as the most promising for the control of gastrintestinal nematodiasis in domestic animals (Terril et al., 2004; Dias et al., 2007a). However, in order to be used as a biological control agent, these nematophagous fungi must have ability for nematode capture and survive passage through gastrointestinal tract (Waller et al., 1994).

Sodium alginate based formulations containing *D. flagrans* mycelial mass have been experimentally evaluated against parasitic nematodes of animals in laboratory and field conditions (Araújo and Sampaio, 2000; Araújo et al., 2000, Dias et al., 2007b). However, none these formulations has been developed to be used in the control of parasitic nematodes of horses in the field.

The objective of the present study was to test a formulation pelletized in sodium alginate containing *D. flagrans* for the biological control of cyathostomin of horses raised in fields.

2. Material and methods

2.1. Organisms

D. flagrans isolate (AC001), nematode-trapping fungus belonging to the genus *Duddingtonia,* was kept in test tubes at 4°C containing 2% corn-meal-agar (2% CMA) in the dark. This isolated originated from a Brazilian soil was obtained by the soil sprinkling technique (Duddington, 1955), modified by Santos et al. (1991).

For induction of fungal mycelia, culture disks, approximately 5 mm in diameter, from fungal isolates in 2% CMA were transferred to 250 mL Erlenmeyer flasks with 150 mL liquid potato-dextrose medium (Difco), pH 6.5, incubated under agitation (120 rpm), in the dark at 26°C, for ten days. Mycelia were then removed for preparation of pellets using sodium alginate as described by Walker and Connick (1983) and modified by Lackey et al. (1993).

2.2. *In vivo* experimental assay

The experiment was conducted at the horse experimental sector of the Federal University of Viçosa, Viçosa, MG-Brazil, latitude 20°45'20" S, longitude 42°52'40" W, from May to October 2007.

In the beginning of the experiment, 3-18 year old crossbred females were previously dewormed by oral administration of 200 µg/kg live weight Ivermectin 1% and 6.6mg /kg live weight Pyrantel Pamoate (Centurion Vallé®, Montes Claros-Minas Gerais, Brasil).

Fourteen days after the antihelmintic treatment, mares were divided into two groups (fungus-treated and control) of eight animals each, with basis on the age and weight of animals. Mean age and mean weight of the fungus-treated group were 6.3 (±6.1) and 386.2 (±54.07) respectively, and 7.1 (± 4.7) and 381.1 (±53.91) for the control group respectively. Mares were placed in two 2.5ha pastures of *Cynodon* sp., naturally infested with equine cyathostomin larvae, which had been previously grazed by young and mature horses. Afterwards, each animal of the treated group received 1 gram pellets/10kg live weight, containing *D. flagrans* mycelial mass mixed with 100 grams of horse commercial ration, twice a week according to procedure described by Assis and Araújo (2003). *D. flagrans* was administered during 6 months from May 2007. Animals of control group received 1gram pellets/10kg live weight without fungus. From the beginning (May) to the end (October) of the experiment, animals from both groups were monthly weighed.

During the experiment the animals were fed daily with 2 kg of horse commercial ration with 14% soybean meal, 83.1% corn meal, 14.5% salt, 1.5% limestone and 14% protein.

Once a week, after the mares had been moved to the pastures, fresh feces were collected directly from the rectum, 72 h after the treatment, for egg count per gram of feces (EPG), according to Gordon and Withlock (1939) and modified by Lima (1989).

Coprocultures were established simultaneously with EPG counts; 20g of feces were mixed with ground, moistened and autoclaved industrial vermiculite (NS Barbosa Ind. Com. ®) and taken to an oven at 26°C, for 8 days, to obtain cyathostome larvae. Larvae were then identified to the genus level as described by Bevilaqua et al. (1993). 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).

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

Every fifteen days, two pasture samples were collected from both treated and control groups, from each pasture, in a zigzag pattern from several and alternated points, 0-20 and 20-40 cm away from fecal pats, in each pasture of the different groups, according to Amarante et al. (1996). Pasture sample collections were always carried out in the morning at 8am. Then, a 500g pasture sample was weighed, and parasitic nematode larvae were recovered according to Lima (1989). The samples were incubated in a drying oven at 100°C, for three days, to determine dry matter. Data were transformed into larvae/kg of dry matter.

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

The egg count curves (EPG) originated from the coprocultures, number of infective larvae recovered from pasture (L_3), correlation between EPG and recovered L_3 and animal weight were compared over the period of the experiment. Data were transformed in log (x+1) and then examined by analyses of variance (ANOVA), followed by Tukey's multiple comparison test with 1% probability. The analyses were performed using the BioEstat 3.0 Software (Ayres et al., 2003).

3. Results

Figure 1 shows the monthly mean EPG counts. EPG of animals treated with *D. flagrans* was lower than the control group, especially in the last four months of the experiment, with significant difference ($p<0.01$), in which the EPG monthly mean of the treated group was 46.2 % lower than the control group. July, August, September and October showed smaller percentages of EPG reduction for fungus treated animals than the control group; 35.4%, 73.2%, 64.3% and 30.5%, respectively. Moreover, fungus-treated animals had EPG values lower than the control group throughout the experiment. Figure 2 shows coproculture data. There was significant difference ($p<0.01$) between the results of fungus-treated animals and the control group in the last four months of the experiment (July, August, September and October) with larval reduction of 57.2%, 59.4%, 68.5% and 51% respectively.

Weights of animals from both groups are shown in Figure 6. There was no significant difference ($p>0.01$) for animal weight during the three first months of the year (May, June and July) between the two groups. However, in the last three months of the experiment (August, September and October), significant differences ($p<0.01$) of 9.74%, 10.26% and 12.21%, respectively, were found for the weight between treated and non-treated animals.

4. Discussion

According to Amarante et al. (1996), EPG counts constitute a parameter that allows evaluation of infection levels in animals and pasture infestation levels by gastrointestinal nematode parasites. A number of studies on *D. flagrans* using horses and ruminants recorded average monthly EPG counts lower for treated animals than for the control group (Baudena et al. 2000b; Knox and Faedo, 2001; Fontenot et al., 2003; Araújo et al., 2006; Paraud et al., 2007). The efficacy of *D. flagrans* application on gastrointestinal of ruminants was also demonstrated in the work of Dimander et al. (2003). These findings are in agreement with results obtained in the present work, confirming that the fungus acts on the infective forms in the fecal environment, with consequently decrease in EPG. There is nevertheless a lack of studies involving nematophagous fungi and equine cyathostomin (Bird and Herd, 1985; Baudena et al., 2000b).

Results seen in Figure 2 suggest that there was a direct action of *D. flagrans* on the infective cyathostomin larvae present in the pasture and a consequent lower parasitic infection of fungus-treated animals (Baudena et al., 2000a; Waghorn et al., 2003; Araújo

et al., 2006). Only the occurrence of small strongyles (Cyathostominae) was observed after the coprocultures, following the parameters described by Bevilaqua et al (1993). Silva et al. (1993) reported that the subfamily Cyathostominae has a high prevalence in a large part of the Brazilian territory, and Carvalho et al. (1998) identified nineteen species of small strongyles in necropsied horses from the state of Minas Gerais. The importance of these parasites for horses is directly related with larval cyathostomosis, a potentially fatal syndrome in most cases, and the high resistance of most gastrointestinal nematode parasites to routine antihelminthics (Reinemeyer, 1986, Reinemeyer et al., 1986).

The number of larvae recovered in the distances 0-20 cm and 20-40 cm from fecal pats (Figure 3) is likely to be directly related with the use of nematophagous fungi that act directly on the L3 present in pastures, making it clear that *D. flagrans* was responsible for the satisfactory reduction of environmental contamination (Araújo et al., 2004).

In a work carried out to evaluate the survival and migration of cyathostomin in Tifton 85 grasses (*Cynodon* spp.) at three collection times (8:00 a.m., 1:00 p.m. and 5:00 p.m.), Bezerra et al. (2007) recorded the largest number of recovered cyathostomin at 8:00 a.m., however no statistical difference was found ($p<0.01$) among the three times. Langrová et al. (2003), in a similar study accomplished in the Czech Republic, reported difference among collection times, with a higher cyathostome recovery at 8:00, 7:00 and 6:00 a.m. respectively. According to Hasslinger and Bittner (1984), temperature and moisture in the mornings favor the high number of L_3 recovered from pastures. In the present work, the largest number of infective larvae recovered was within the distance 0-20 cm away from the fecal pats. This result agrees with findings reported by Quinelato et al. (2008) and Dias et al. (2007b) who recorded larger numbers of larvae recovered within 0-20 cm from fecal pats, confirming that few larvae that leave the feces migrate to the grass beyond 0-20 cm. According to Stromberg (1997), temperature and moisture are essential for the development of infective larvae. Only cyathostomin larvae were found in the grass over the experimental period (May to October). Climatic conditions, such as temperature, relative humidity and rainfall favored the development of free-living stages and migration to the grass, Figures 4 and 5. The lowest rainfall rates occurred in July and August with 12.64 and 16.96 mm^3 respectively, however, larval count was high owing to accumulated larval loads. June and September had the highest rainfall rates, 25.25 and 35.31 mm^3 respectively, and the smallest larval number was recorded, possibly because the L_3 were washed off by rain (Figures 3 and 5). Quinelato et al. (2008), working in tropical southeast Brazil, reported higher recovery of cyathostomin larvae from grass and later from feces in the dry period, and that the environmental conditions were favorable

for recovering these larvae. The authors also argued that horses might be infected during the whole year in tropical climates, since L_3 are always present in the pastures and, besides, the grass type can affect their recovery. Langrová et al. (2003), in central Europe, suggested that L_3 respond to rain through dispersion within the vegetation, existing a moderate correlation between moisture and the L_3 number in the pasture.

Courtney (1999) observed that during the dry period L_3 development is slower, but they survive longer. Still, Fernández et al. (1997) and Baudena et al. (2000a) argued that possibly the survival of these parasites in the environment is strongly related with temperature and that few larvae would be found in feces in the summer. Baudena et al. (2000a) recorded field data in southern Louisiana, a region with subtropical climate in The United States, which suggested that months with milder temperature would have larger number of infective larvae present in the pasture. Such information is in accordance with the results found in this work in which the largest number of larvae recovered in pastures was found during months of milder temperature (Figure 3). Peña et al. (2002) and Chandrawathani et al. (2004) reported *D. flagrans* being able to reduce more than 90% of infective larvae present in fecal pats of ruminants.

Fontenot et al. (2003) also discussed that besides *D. flagrans* decreasing infectious forms of gastrointestinal nematode parasites in pastures, it would avoid contamination of new animals entering these sites.

The correlation coefficient between EPG and infective larvae recovered from pastures of group 1 within 0-20 cm from fecal pats was 0.0662; and for the distance 20-40 cm was 0.0416. For group 2, the correlation coefficient between EPG and infective larvae recovered within 0-20 cm from the fecal pats was –0.0394 and within 20-40 cm was 0.0401. These results showed weak, non-significant correlations, close to zero, nevertheless, as Dias et al. (2007b) 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).

D. flagrans is considered the most promising species in for biological control against gastrointestinal nematode parasites of livestock (Faedo et al., 2002). It has been used successfully in several laboratory and field studies (Araújo et al., 2006). Baudena et al. (2000b) proved the effectiveness of *D. flagrans* to reduce recovery of cyathostomin larvae from pastures, recording decrease in percentage of recovered larvae in the treated group compared with the control group, after receiving 2×10^6 spores per kilogram of live weight during 4 days.

In a work carried out with two fungal isolates of the genus *Monacrosporium*, Assis Araújo (2003) found fungal mycelia in horse feces up to 96 hours after passing through the gastrointestinal tract of horses. Therefore, in this work, for an efficient weekly coverage, *D. flagrans* was applied twice a week.

In Malaysia, the effectiveness of daily administration of *D. flagrans* to sheep was confirmed by Chandrawathani et al. (2003). Terrill et al. (2004) also reported reduction of larvae in feces of goats infected with predominantly *H. contortus*. They also found that the daily administration of fungi (*D. flagrans*) was more effective than every two or three days. The frequency of treatments in this work promoted reduction of pasture contamination, mainly the weekly treatment.

The difference ($p<0.01$) observed in the weight gain of the treated animals compared to the control group was probably due to lower parasite load of animals that received pellets containing mycelial mass of the nematophagous fungus *D. flagrans*, which may have contributed to a better food conversion of treated animals. These results are similar to those reported by Dias et al. (2007a) on weight gain of cattle treated with pellets containing mycelial mass of *D. flagrans*.

The findings reported in this study suggest that the nematophagous fungus *D. flagrans* could be used in an integrated program to control horse cyathostomin in southeastern Brazil. It would be useful to carry out a previous anthelmintic treatment to decrease parasite load in animals and therefore the EPG, and starting from that to supply animal feed added with the fungus to control the larval forms present in the environment and thus prevent reinfection.

4. Conclusion

Treatment of horses with pellets containing mycelial mass of the nematophagous fungus *D. flagrans* can be effective to control cyathostomin in tropical southeastern Brazil.

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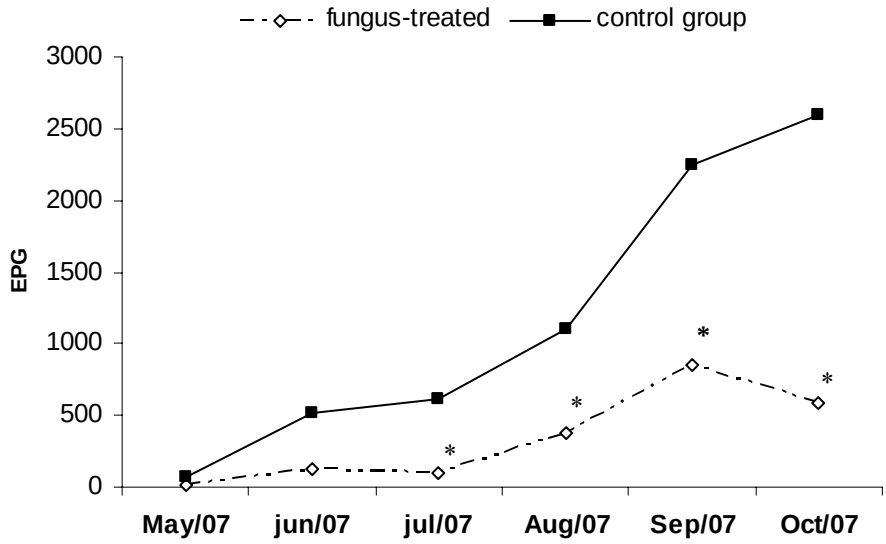

Fig.1 Monthly means of eggs per gram of feces (EPG) of fungus-treated and control animals collected from May to October 2007, Viçosa – MG – Brazil. Significant difference ($p<0.01$) between the treated group and the control denoted by asterisk- Tukey test.

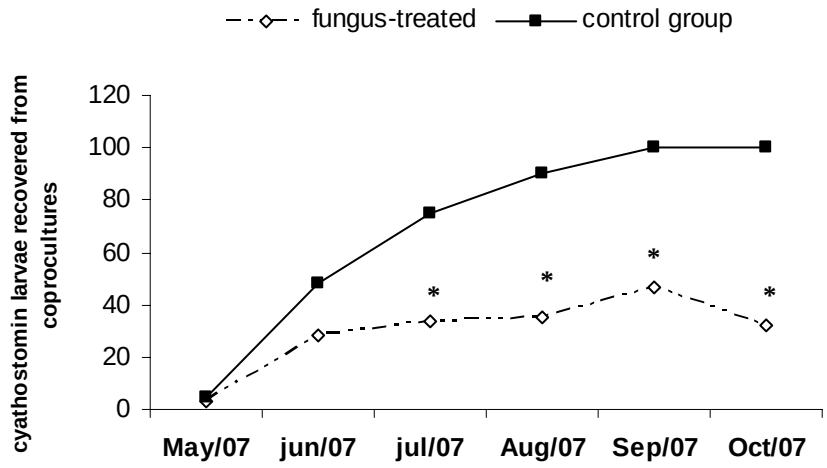

Fig.2 Mean monthly number of cyathostomin larvae recovered from coproculture of fungus-treated horses and control group collected from May to October 2007, Viçosa – MG – Brazil. Significant difference ($p<0.01$) between the treated group and the control denoted by asterisk- Tukey test.

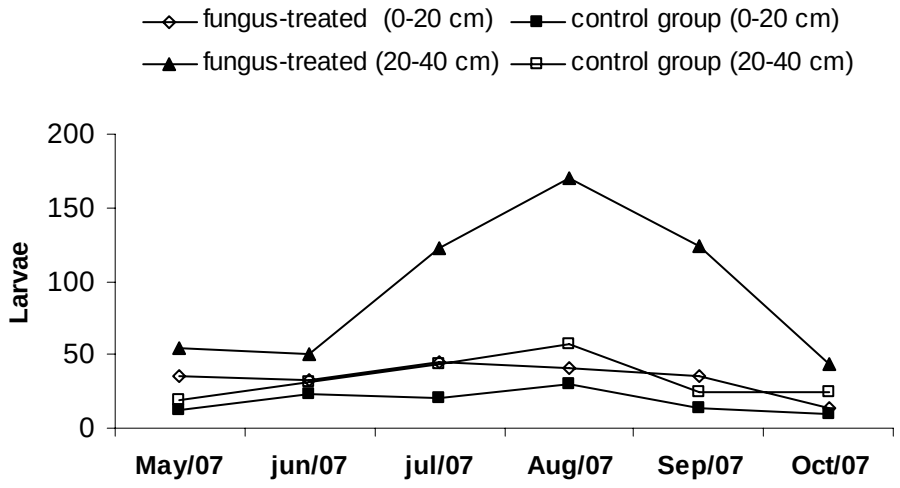

Fig.3 Monthly counts of number of infective nematode larvae per kilogram of dry matter recovered from pastures of fungus-treated horses and control collected in sampling distances up to 20 cm and 20-40 cm from fecal pats, from May to October 2007, Viçosa – MG- Brazil.

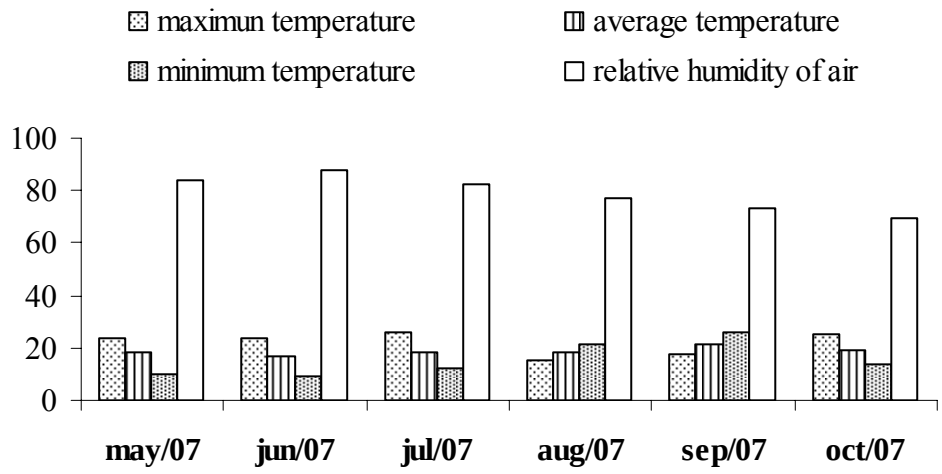

Fig.4 Averages of maximum, average and minimum monthly temperatures (ºC) and air relative humidity (%) recorded from May to October 2007, Viçosa – MG- Brazil.

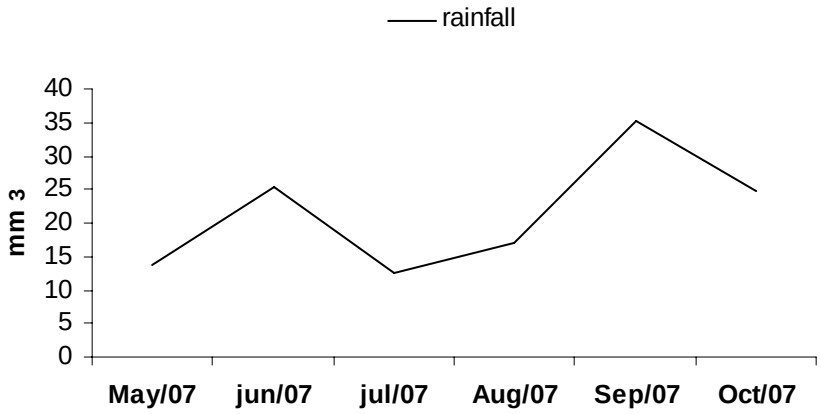

Fig.5 Monthly rainfall (mm^3) recorded from May to October 2007, Viçosa – MG- Brazil.

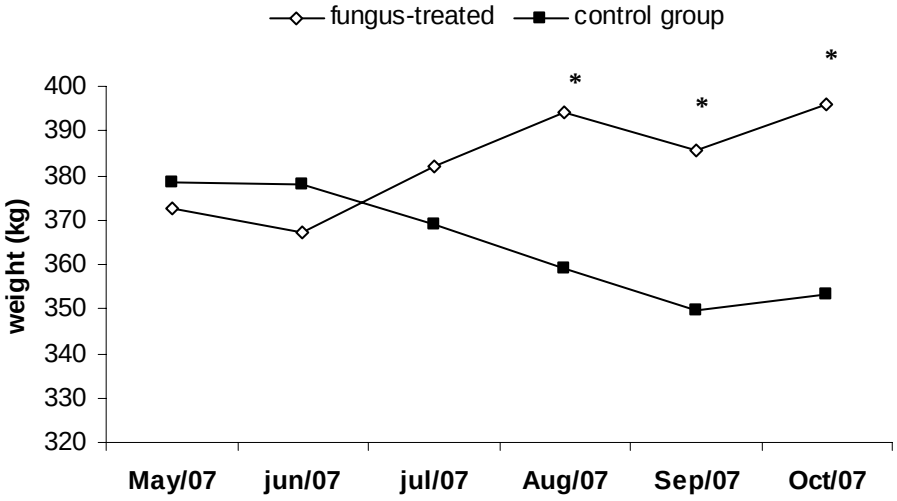

Fig.6 Monthly means of weight (kg) of fungus-treated horses and control from May to October 2007, Viçosa – MG- Brazil. Significant difference ($p<0.01$) between the treated group and the control denoted by asterisk- Tukey test.

CAPÍTULO 2

Duddingtonia flagrans, Monacrosporium thaumasium* and *Pochonia chlamydosporia* as possible biological control agents *of Oxyuris equi* and *Austroxyuris finlaysoni

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Abstract

The action of four fungal isolates of the species *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34a) and *Pochonia chlamydosporia* (VC1 and VC4) on eggs of *Oxyuris equi* and *Austroxyuris finlaysoni* was evaluated in two assays (A and B). Eggs of *O. equi* (Test A) and *A. finlaysoni* (Test B) were plated on Petri dishes with 2% water-agar with grown fungal isolates and control without fungus. After five, 10 and 15 days, one hundred eggs were collected and classified according to the following parameters: type 1 effect, physiological and biochemical effect without morphological damage to the eggshell; type 2 effect, lytic effect with morphological alteration of the eggshell and embryo, and type 3 effect, lytic effect with morphological alteration of the eggshell and embryo, hyphal penetration and internal egg colonization. *P. chlamydosporia* isolates VC1 and VC4 showed ovicidal activity for type 1, 2 and 3 effects on eggs of *O. equi* and eggs of *A. finlaysoni*. *In vitro* assays A and B showed that *P. chlamydosporia* had negative influence on eggs of *O. equi* and *A. finlaysoni* and can be considered as a potential biological control agent of nematodes.

1. Introduction

O. equi is a common inhabitant of the lower colon or occasionally the large colon of horses. Urquhart *et al*. (1998) and Bowman *et al*. (2006) discussed that most pathogenic effects of this parasite in the intestine are caused by the feeding habits of third and fourth stage infective larvae, resulting in small erosions in the mucosa. However, a more important effect is the irritation caused by adult females during oviposition. Bowman *et al*. (2006) also stated that pregnant female parasites instead of eliminating their eggs in the fecal bulk migrate to the lower part of the colon and rectum and out of the anus to adhere egg masses (8 to 60 thousand eggs) in the anal skin and the perianal region, causing anal itching.

The commercial raising of wild animals in countries with large regional differences has been recognized as an important protein source for subsistence of poor populations. There are however some important barriers to the commercial production of these animals, particularly gastrointestinal nematodiosis (Bonuti *et al*., 2002). *Austroxyuris finlaysoni*, a marsupial Oxyuridae, is a gastrointestinal nematode parasite of Petauroides volans (Petauridae, Marsupiala). Marsupials are primitive mammals only found in the Americas and Australia. Gomes *et al*. (2003) reported that some marsupials, for instance, the white-eared opossum (*Didelphis albiventris*), have great medical-veterinary importance as reservoir for gastrointestinal nematode parasites and definitive host of potentially zoonotic intracellular parasites with wide distribution in neo-tropical regions. Studies on marsupial endoparasites are scarce and generally aimed at helminth systematic (Hugot & Bougnoux, 1988).

Alternative treatments may help to reduce the continued use of the same class of antihelminths as well as doses above the recommendation. Biological control carried out with natural nematode antagonists, such as nematophagous fungi, is among these alternatives. These fungi are biologically very important in the environment, playing a role in recycling carbon, nitrogen and other elements derived from nematode degradation (Araújo *et al*., 2004).

These organisms comprise various types of fungi characterized as predators, endoparasites and opportunists, their action is concentrated in the fecal environment and aimed against free-living parasite larvae. Predatory fungi and egg parasites are certainly the most studied groups and show the greatest biological control potential (Nordbring-Hertz *et al*., 2002). In the predatory group, *Duddingtonia flagrans* and *Monacrosporium thaumasium* are promising species in the control of gastrointestinal nematode parasites of domestic animals. *Pochonia chlamydosporia*, in the ovicidal group, stands out as an

important biological control agent of parasite eggs (Araújo *et al*., 2008; Braga *et al*., 2008).

This study evaluated the *in vitro* effect of the nematophagous fungi *D. flagrans*, *M. thaumasium* and *P. chlamydosporia* on eggs of *O. equi* and *A. finlaysoni* in two experimental assays (A and B) in intervals of five, 10 and 15 days of interaction.

2. Material and methods

Four nematophagous fungal isolates, (AC001) from *D. flagrans*, (NF34a) from *M. thaumasium* and (VC1 and VC4) from *P. chlamydosporia*, were kept in test tubes containing 2% corn-meal-agar (2% CMA), at 4°C in the dark 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, Brazil.

Culture disks, 4mm in diameter, were extracted from the fungal cultures kept in test tubes containing 2% CMA and transferred to 9.0cm in diameter Petri dishes containing 20 ml of 2% potato-dextrose-agar (2% PDA), at 26°C, in the dark for 10 days. After fungal growth, new 4-mm culture disks were transferred to 9.0cm diameter Petri dishes containing 20 mL of 2% water-agar (2% WA) and kept at 26° C in the dark for 10 days.

Eggs of *O. equi* were recovered by dissection of adult female specimens from feces of an infected horse and identified according to Urquhart *et al*. (1998).

Eggs of *A. finlaysoni* were recovered by dissection of adult specimens obtained from the autopsy of a white-eared opossum (*D. albiventris*) that had died of natural causes. Adult parasites were identified according to Johnston & Mawson (1938) apud Hugot & Bougnoux (1988).

The work was divided into two experimental tests carried out in different stages during 15 days. The fungal isolates *D. flagrans*, *M. thaumasium* and *P. chlamydosporia* exerted action against *O. equi* and *A. finlaysoni* eggs at five, 10 and 15 days according to Araujo *et al.* (2008), in Petri dishes (9cm diameter) containing only 2% WA medium, with ten repetitions per group. Each Petri dish contained a thousand eggs with one fungal isolate. The control group contained a thousand eggs without fungi. At five, 10 and 15 days post-inoculation, a hundred eggs were removed from each plate containing the fungal isolates and from the control (without fungi) as described by Araújo *et al*. (1995). The eggs were placed on glass slides with a drop of 1% Amam blue and evaluated under light microscopy (40x objective lens) according to Lysek *et al*. (1982): type 1 - physiological and biochemical effect with no morphological damage to the eggshell and with hyphal

adherence; type 2 - lytic effect with morphological changes in eggshells and embryos, without hyphal penetration; and type 3 - lytic effect with morphological change in eggshells and embryos, with hyphal penetration and internal egg colonization.

Data were examined by the nonparametric Friedman test at 1% probability level (Ayres *et al*., 2003).

3. Results and Discussion

Table 1 shows the percentage results of fungal effect on *O. equi* eggs at five, 10 and 15 interaction days. The fungal isolate *D. flagrans* AC001 showed 54.5%, 58.6% and 62.0% of type 1 effect on *O. equi* eggs at five, 10 and 15 days, respectively. Type 2 and 3 effects were not detected for this isolate in the studied periods. However, only AC001 hyphae were found adhered to *O. equi* eggshell, characterizing effect type 1. Isolate *M. thaumasium* (NF34a) had percentage results similar to AC001; 61.3%, 62.7% and 69.0% of only type 1 effect at five, 10 and 15 days, respectively.

No significant difference was found ($p>0.01$) for type 1 effect when comparing the effect of isolates AC001 and NF34a on *O. equi* eggs.

Isolates VC1 and VC4 of *P. chlamydosporia* showed type 3 effect on *O. equi* eggs in the three studied intervals. In this experiment, VC1 showed percentage results for this effect of 27.2%, 23.1% and 25.0% at five, 10 and 15 days respectively. Similarly, VC4 showed percentages of 13.6%, 25.4% and 21.8% for type-3 effect in the same intervals.

VC1 also showed results for type 1 and 2 effects, with percentages of 18.6%, 21.8% and 19.5% at five, 10 and 15 days for type 1 effect respectively; and 24.0%; 23.6% and 26.1% for type 2 effect. VC4 presented percentage results for type 1 effect of 25.0%, 24.5% and 25.1% and for type 2 effect of 26.3%, 24.5% and 28.6% at five, 10 and 15 days, respectively. No differences ($p>0.01$) were found between VC1 and VC4 for types-1, 2 and 3 effects on *O. equi* eggs during the interaction days.

Table 2 shows the percentage results for the effect of different fungi on *Austroxyuris finlaysoni* eggs at five, 10 and 15 interaction days. *D. flagrans* AC001 showed percentages results of 55.9%, 60.0% and 59.0% for type 1 effect on *A. finlaysoni* eggs at five, 10 and 15 days, respectively, but no results for type 2 and 3 effects, without egg damage. Therefore, *D. flagrans* was not classified as ovicidal. *M. thaumasium* (NF34a) showed results similar to AC001; only type 1 effect with percentage results of 52.2%, 61.8% and 70.4% at five, 10 and 15 days respectively, also showing no ovicidal activity.

Comparison of AC001 and NF34a for type 1 effect on *A. finlaysoni* eggs showed no significant difference ($p > 0.01$), both had the same action.

The isolate VC1 showed type 1, 2 and 3 effects at five, 10 and 15 interaction days, respectively, on *A. finlaysoni* eggs. Ovicidal activity presupposes fungi have 3 effects of 16.8%, 27.0% and 21.0 % at five, 10 and 15 days, respectively; 25.9%, 29.5% and 28.6% for type 1 effect; and 27.2%, 22.2% and 23.9% for type 2 effect. Isolate VC4 also showed type 3 effect of 19.0%, 20.4% and 17.0%; type 1 effect of 16.3%, 30.0% and 34.0%; and type 2 effect of 26.3%, 20.4% and 20.8% at five, 10 and 15 days respectively.

No differences ($p>0.01$) were found between VC1 and VC4 for type 1, 2 and 3 effects on *A. finlaysoni* eggs, demonstrating that there was no difference between their forms of interaction with *A. finlaysoni* eggs.

This work confirmed the ovicidal activity of *P. chlamydosporia* isolates (VC1 and VC4) on *O. equi* and *A. finlaysoni* eggs, as well as the capacity of interaction of predatory fungal isolates AC001 and NF34a with parasite eggs.

Biological control using nematophagous fungi, biologically very important in the environment, is a promising strategy in the control of gastrointestinal nematode parasites in wild and domestic animals (Larsen, 1999; Sanyal *et al.,* 2008). Nematophagous fungi have predatory capacity against L_3 of gastrointestinal nematode parasites, specially the genera *Duddingtonia* and *Monacrosporium* (Araújo *et al*., 2004; 2007). Ovicidal nematophagous fungi have been successfully tested as biological control agents of gastrointestinal nematode parasites eggs, mainly the genus *Pochonia* (Braga *et al*., 2007; Araújo *et al.* (2008). Although some studies have shown the action of the nematophagous fungi *Duddingtonia*, *Monacrosporium* and *Pochonia* as biological control of gastrointestinal nematodiosis of domestic animals (Araújo *et al*., 2008), there are no reports demonstrating their action on eggs of *O. equi* and gastrointestinal nematodes of wild animals such as *A. finlaysoni*.

The appressorium was the first mechanical structure formed by ovicidal fungi, allowing adhesion to the host surface and providing mechanical strength for egg penetration (Lysek *et al*., 1982). According to Stirling & West (1991), the direct effect of parasitism of this fungus on embryo development is the enzymatic action on the eggshell increasing its permeability and favoring the passage of toxins. As a biological control agent, a fungus has to survive the passage through the gastrointestinal tract of animals, such as the chlamydospores producing species *D. flagrans* and *P. chlamydosporia* (Braga *et al*., 2008).

Morgan-Jones *et al*. (1983) suggested that the ideal procedure would be to feed animals with eggs, after the prior contact of 15 days, to verify whether or not eggs and embryos become unviable.

As suggested by Araújo *et al*. (1995), the experimental tests were carried out with longer intervals of interaction between fungi and parasite eggs to observe a possible type 3 effect. After 15 days, nevertheless, hyphae and conidia of isolates AC001 and NF34a were found colonizing only the egg surface, characterizing type 1 effect. Isolates VC1 and VC4 produced hyphae adhering to the surface and colonizing inside the eggs, and, later, egg rupture was also observed, characterizing type 3 effect.

According to Lysek *et al*. (1982) the ovicidal conditions of a fungus is characterized by the type 3 effect during egg infection; and as pointed by Gams & Zare (2001) mechanical and enzymatic actions are involved in the process.

Differences in the interaction of *P. chlamydosporia* with eggs of some helminth genera have been attributed to peculiarities of these eggs. For instance, eggs of *F. hepatica* and *S. mansoni* are large, with diameters between 150µm and 190µm and very resistant (Burger & Stoye, 1978). This could reduce the ovicidal activity of *P. chlamydosporia* (Braga *et al*., 2008). Eggs of *A. lumbricoides* and *A. sum* have a thick chitin-protein capsule (Silva & Massara, 2005; Bowman *et al*., 2006). This is important information, since *P. chlamydosporia* has enzymatic and mechanical action on eggs during the interaction days, and eggs without capsule would undergo less damage when in contact with the fungus.

The penetration mechanism of ovicidal fungi on parasitized eggs is not yet fully understood. Still, several authors admit that the enzymatic activity is a key component in the attack and penetration of eggs by fungi (Lysek & Sterba, 1991).

Results of the experiment showed that *P. chlamydosporia* (VC1 and VC4) negatively influenced eggs of *O. equi* and *A. finlaysoni*, and is considered as a potential candidate for the biological control of nematodes.

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Table 1 Percentages and standard deviations of the ovicidal activity of nematophagous fungi *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34a), *Pochonia chlamydosporia* (VC1 and VC4) and control group without fungi on eggs of *Oxyuris equi* at five, 10 and 15 interaction days.

Table 2 Percentages and standard deviations of the ovicidal activity of nematophagous fungi *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34a), *Pochonia chlamydosporia* (VC1 and VC4) and control group without fungi on eggs of *Austroxyuris finlaysoni* at five, 10 and 15 interaction days.

Isolates	Effect at five days of interaction		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
AC001	54.5^{A} ±21.8	0^{B}±0	0^{B}±0
NF34a	61.3^{A}±22.0	0^{B}±0	0^{B}±0
VC1	18.6^{B}±11.8	24.0^{A}±12.0	27.2^{A}±12.5
VC4	25.0^{B}±11.4	26.3^{A}±10.9	13.6^{A}±10.2
Control	0^{C}±0	0^{B}±0	0^{B}±0

Isolates	Effect at 10 day		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
AC 001	58.6^{A}±21.3	0^{B}±0	0^{B}±0
NF34a	62.7^{A}±22.1	0^{B}±0	0^{B}±0
VC 1	21.8^{B}±10.0	23.6^{A}±9.5	23.1^{A} ±10.5
VC 4	24.5^{B}±9.8	24.5^{A}±10.5	25.4^{A}±9.6
Control	0^{C}±0	0^{B}±0	0^{B}±0

Isolates	Effect at 15 day		
	Effect Type 1*	**Effect Type 2****	**Effect Type3*****
AC001	62.0^{A}±21.9	0^{B}±0	0^{B}±0
NF34a	69.0^{A}±24.0	0^{B}±0	0^{B}±0
VC1	19.5^{B}±11.0	26.1^{A}±9.8	25.0^{A}±10.2
VC4	25.1^{B}±13.7	28.6^{A}±12.0	21.8^{A}±10.6
Control	0^{C}±0	0^{B}±0	0^{B}±0

Percentages followed by same letter in the same column are not significantly different (P>0.01) – Friedman test.

Physiological, biochemical effect without morphological damage to eggshell, with hyphae adhered to the shell

**Lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell.

***Lytic effect with morphological alteration of embryo and eggshell, besides hyphal penetration and internal colonization

Isolates	Effect at five day		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
AC001	55.9^{A}±20.9	0^{B}±0	0^{B}±0
NF34a	52.2^{A}±25.5	0^{B}±0	0^{B}±0
VC1	25.9^{B}±11.5	27.2^{A}±8.7	16.8^{A}±10.3
VC4	16.3^{B}±9.7	26.3^{A}±10.2	19.0^{A}±11.5
Control	0^{C}±0	0^{B}±0	0^{B}±0

Isolates	Effect at 10 day		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
AC 001	60.0^{A}±20.3	0^{B}±0	0^{B}±0
NF34a	61.8^{A}±22.3	0^{B}±0	0^{B}±0
VC1	29.5^{B}±11.7	22.2^{A}±9.3	27.0^{A}±10.3
VC4	30.0^{B}±11.2	20.4^{A}±8.8	20.4^{A}±7.5
Control	0^{C}±0	0^{B}±0	0^{B}±0

Isolates	Effect at 15 day		
	Effect Type 1*	**Effect Type 2****	**Effect Type3*****
AC001	59.0^{A}±24.1	0^{B}±0	0^{B}±0
NF34a	70.4^{A}±25.0	0^{B}±0	0^{B}±0
VC1	28.6^{B}±14.5	23.9^{A}±10.3	21.0^{A}±12.3
VC4	34.0^{B}±14.4	20.8^{A}±8.7	17.9^{A}±13.2
Control	0^{C}±0	0^{B}±0	0^{B}±0

Percentages followed by same letter in the same column are not significantly different (P>0.01) – Friedman test.

Physiological, biochemical effect without morphological damage to eggshell, with hyphae adhered to the shell

**Lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell.

***Lytic effect with morphological alteration of embryo and eggshell, besides hyphal penetration and internal colonization

CAPÍTULO 3

Viability of the nematophagous fungus *Pochonia chlamydosporia* after passage through the gastrointestinal tract of horses

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Abstract

The predatory capacity of the nematophagous fungus *Pochonia chlamydosporia* (isolate VC4) embedded in sodium alginate pellets after passage through the gastrointestinal tract of horses was assessed *in vitro* against *Oxyuris equi* eggs. Twelve previously dewormed crossbred mares, average weight of 362.5 kg (± 21) were used in the experiment. Each animal of the treated group received an oral dose (100g) of sodium alginate pellets containing *P. chlamydosporia* mycelial mass. The control group received pellets without fungus. Faecal samples from fungus-treated and control groups were collected at intervals of 8, 12, 24, 36, 48 and 72 hours after pellet administration and placed in Petri dishes containing 2% water-agar. One thousand eggs of *O. equi* were plated in Petri dishes of both treated and control groups, with six replicates, and incubated in oven, 25^{o}C, in the dark, for 30 days. At the end of the experiment, one hundred eggs were removed from each Petri dish and classified according to the following parameters: type 1, physiological and biochemical effect without morphological damage to eggshell, with hyphae adhered to the shell; type 2, lytic effect with morphological change in the eggshell and embryo without hyphal penetration, and type 3, lytic effect with morphological change in the eggshell and embryo, with hyphal penetration and internal egg colonization. Chlamydospore production was observed in Petri dishes of the treated group. The isolate VC4 remained viable after passing through the gastrointestinal tract of horses and maintained the ovicidal activity against *O. equi* eggs when compared with the control group ($p < 0.01$) after each collection interval: 29.1% (8 hours), 28.2% (12 hours), 31.1% (24 hours), 27.4% (36 hours); 30.9% (48 hours) and 28.4% (72 hours). The results suggest that *P. chlamydosporia* could be used as an effective biological control agent of *O. equi* eggs in natural conditions.

Keywords: Nematophagous fungus; *Pochonia chlamydosporia*; *Oxyuris equi;* biological control.

1. Introduction

The helminth fauna of horses has a large variety of parasitic organisms with species and genera of great interest, including *Parascaris equorum*, *Anoplocephala perfoliata*, *Oxyuris equi*, *Cyathostomum* spp., and *Strongylus* spp (Kaplan, 2002).

O. equi was reported as early as the the 5th century BC by Hipocrates as a common inhabitant of the lower colon, or occasionally the large colon, of horses (Morgan and Hawkins, 1949). According to Urquhart et al. (1998) and Bowman et al. (2006), the main pathogenic effects of this parasite in the intestine are related to the feeding habits of third- and fourth-stage infective larvae, causing small mucoal erosions. However, a more important effect is the irritation caused by adult females during egg laying. Fertilized females migrate down to the colon and rectum and crawl out of the anus, deposit egg masses (8 to 60 thousand eggs) on the skin near the anal opening and perianal region causing intense anal and perineal pruritis (Bowman et al., 2006).

The use of anthelmintic drugs has been the conventional approach to controlling worms in horses. But these drugs have not been totally effective for the control of gastrointestinal nematode parasites, since their action is restricted to adult forms and there is occurrence of resistance (Kaplan, 2002; Matthews et al., 2004).

The continued use of the same anthelmintic class, as well as the rapid rotation between compound groups, introduction of resistant worms and the use of doses lower than the recommendation should be avoided. Biological control using natural nematode antagonistic fungi is among the most viable alternatives (Mota et al., 2003; Araújo et al., 2008). These organisms comprise different types of fungi classified into predators, endoparasites and ovicidal, whose action is concentrated in the faecal environment and directed against parasitic eggs and free-living larvae (Jatala, 1986; Braga et al., 2009a).

Ovicidal fungi produce chitinolytic enzymes that are potentially involved in egg infection (Kerry and Hidalgo, 2004). Lysek et al. (1982) define that the ovicidal capacity of a fungus is characterized by the presence of the type 3 effect (ovicidal activity) with the destruction of parasitized eggs. In this group, the species *Pochonia chlamydosporia* stands out for its appressorial formation developed from undifferentiated hyphae that colonize eggshell and penetrate the egg causing its destruction (Lysek et al., 1991; Braga et al., 2007; Araujo et al., 2009).

This fungus is a facultative deuteromycete parasite of nematode eggs with wide distribution. It is characterized by its rapid growth in laboratory conditions, producing colonies with diameters between 15-40 μm and different-shaped conidia: elliptical, globose and rod-shaped, as well as small conidiophores and differentiated hyphae, which

can be erect in some situations (Gams and Zare, 2001; De et al., 2008; Braga et al., 2009a, b).

Kerry (2000) and Kerry and Hidalgo (2004) discuss that *P. chlamydosporia* has important characteristics required of a fungus to be used as an effective biological control agent, such as efficient reduction of nematode populations, longevity in the soil and chlamydospores production. Numerous studies have focused on the evaluation of the efficiency of fungal isolates after passage through the intestinal tract of domestic animals (Araújo and Sampaio, 2000; Araújo et al., 2000; Carvalho et al., 2009). However, there have been no reports of studies using sodium alginate formulations containing mycelia of ovicidal fungi, particularly *P. chlamydosporia*.

In this work, *P. chlamydosporia* (isolate VC4) was evaluated *in vivo* for its ability to pass through the gastrointestinal tract of horses, resistance and viability, assessing its predatory capacity against *O. equi* eggs.

2. Material and methods

2.1. Passage Test

The isolate VC4 of the ovicidal fungus *Pochonia chlamydosporia* was kept in test tubes at 4^{o}C containing 2% corn-meal agar (2% CMA) in the dark. The isolate was obtained from a Brazilian soil using the soil sprinkling method (Duddington, 1955) modified by Santos et al. (1991).

Fungal mycelia were obtained by transferring culture disks (approximately 5 mm in diameter) of fungal isolates in 2% CMA to 250 mL Erlenmeyer flasks with 150 mL liquid potato-dextrose medium (Difco), pH 6.5, and incubated under agitation (120 rpm), in the dark at 26^{o}C, for ten days. Mycelia were then removed and pelletized in sodium alginate as described by Walker and Connick (1983) and modified by Lackey et al. (1993).

2.1.2. *Oxyuris equi* eggs

Eggs of *O. equi* were recovered from the dissection of adult female specimens obtained from the faeces of a infected horse and identified according to Urquhart et al. (1998).

2.1.3. Experimental Site

The experiment was conducted at the horse experimental sector of the Federal University of Viçosa, Viçosa, MG-Brazil, latitude 20°45’20” S, longitude 42°52’40” W.

Twelve crossbred mares, average weight of 362.5 kg (± 21.0), which were kept in stables, were previously dewormed with 200 µg/kg live weight Ivermectin 1% and 6.6mg /kg live weight Pyrantel Pamoate (Centurion Vallé®, Montes Claros-Minas Gerais, Brazil).

Fourteen days after the anthelmintic treatment, the mares were randomly separated into two groups of six animals each (fungus-treated and control) and kept in separate stalls.

The animals received water *ad libtum* and were daily fed with autoclaved grass and commercial ration with 14% soybean meal, 83.1% corn meal, 1.4% salt, 1.5% limestone and 14% protein. Each animal of the treated group received 100g pellets, as a single dose, containing *P. chlamydosporia* mycelia mixed in 100g of commercial horse food. Animals of the control group received a single dose of 100 g of sodium alginate pellets without fungus.

Fecal samples of both treated and control groups were collected from the rectal ampoule of each animal at intervals of 8, 12, 24, 36, 48 and 72 hours after fungal and control treatments, as described by Assis and Araújo (2003). The samples were homogenized; and faeces (2g) were removed and placed in Petri dishes, 9cm diameter, containing 2% WA. One thousand eggs of *O. equi* were plated on each Petri dish of the treated and control groups and incubated in oven, at 25^{o}C, in the dark, for 30 days. Six repetitions were carried out for each time interval for both groups. Plates of both fungus-treated and control groups were daily examined to assess characteristic structures of *P. chlamydosporia* (VC4) such as conidia, conidiophores and chlamydospores, which were analyzed according to the classification keys proposed by (Gams and Zare, 2001).

Thirty days after faeces collection, one hundred eggs were removed from each Petri dish of treated and control groups using the technique described by Araújo et al. (1995) and placed on glass slides with a drop of 1% Aman blue. The eggs were examined under a light microscope (40x objective) using the parameters established by Lysek et al. (1982): type 1 effect, physiological and biochemical effect without morphological damage to eggshell with hyphae adhered to the shell; type 2 effect, lytic effect with morphological change in the eggshell and embryo without hyphal penetration, and type 3 effect, lytic effect with morphological change in the eggshell and embryo with hyphal penetration and internal egg colonization.

2.1.4. Statistical analysis

Data were analyzed by the nonparametric Friedman's test at 1% probability level (Ayres et al., 2003).

3. Results

Table 1 show the results for ovicidal activity of *P. chlamydosporia* against *O. equi* eggs at the faeces collection times (8, 12, 24, 36, 48 and 72 hours).

Chlamydospore production of the fungus *P. chlamydosporia* (VC4) was observed in plates of group fungus-treated. Three days after the oral administration of fungal pellets, it was possible to observe conidia formation, confirming the presence of *P. chlamydosporia* in all Petri dishes of samples collected 24 hours after treatment, whereas no fungus was detected in Petri dishes of the control group.

P. chlamydosporia showed ovicidal activity on *O. equi* eggs after passage through the gastrointestinal tract of horses and remained viable at all collection times starting at 8 hours post-incubation. The largest percentages of type 3 effect (ovicidal activity) were observed in the treated group at 24 and 48 hours after pellet administration ($p<0.01$).

Because the 2% WA medium is nutritionally poor, the presence of faeces in the Petri dishes of the fungus-treated group was crucial for conidia production and destruction of *O. equi* eggs by the fungal isolate. The nematophagous fungus was not observed in Petri dishes of the control group during the experiment. Egg destruction was confirmed by the percentage results of the ovicidal activity found at the end of the experiment (Table 1).

The VC4 isolate showed percentage results for effects type 1, 2 and 3 against *O. equi* eggs at all faeces collection times (8, 12, 24, 36, 48 and 72 hours), being therefore considered as a promising control agent. The following percentages were found for type 3 effect (ovicidal activity): 29.1% (8 hours), 28.2% (12 hours), 31.1% (24 hours), 27.4% (36 hours), 30.9% (48 hours) and 28.4% (72 hours).

After 30 days, the statistical analysis showed significant difference ($p <0.01$) between the action of the isolate VC4 in Petri dishes of the treated group and the control group. However, no difference was found ($p> 0.01$) among the collecting times in the treated group.

Light microscopy (40 x objectives) showed hyphal colonization by the isolate VC4 on the surface and inside *O. equi* eggs and egg destruction after 30 incubation days.

4. Discussion

Although there are prospects for using nematophagous fungi in the control of gastrointestinal nematodiosis of domestic animals, the most effective activity has been observed in fresh faeces. Nematode parasites of animals go through egg to larval stages before migrating to the pasture as infective larvae, or, as in some helminth species, the infective stage is developed inside the eggs (Larsen, 1999; Mota et al., 2003).

Therefore, to be considered as an efficient biological control agent, nematophagous fungi must have the ability to survive the passage through gastrointestinal tract of domestic animals and germinate in the faeces (Larsen, 1999; Chandrawathani et al., 2003; Dimander et al., 2003; Kerry and Hidalgo, 2004; Dias et al., 2007; Campos et al., 2008).

Chlamydospore production is also very important for the effective reduction of free-living stages of gastrointestinal nematode parasites in domestic animals (Larsen, 1999; Terrill et al., 2004). In this work, *P. chlamydosporia* (VC4) survived the passage through the gastrointestinal tract of horses, germinated in the faeces and produced chlamydospores in all Petri dishes of treated animals, confirming, in this way, its potential as a biological control agent.

P. chlamydosporia (VC4) has been used only in laboratory conditions (Garcla et al., 2004; Araujo et al., 2009). Some studies have showed its ovicidal activity on eggs of nematode, trematode and cestode parasites of domestic animals and humans (Braga et al., 2007, Araújo et al., 2008, Braga et al., 2008a, b). These studies, nevertheless, have determined its resistance to the passage through the gastrointestinal tract of domestic animals.

In recent work, Braga et al. (2009b) tested the *in vitro* activity of *P. chlamydosporia* (isolates VC1 and VC4) on eggs of *O. equi* and found that both isolates were efficient in egg predation at intervals of five, 10 and 15 days, but no significant difference ($p > 0.01$) was found between their ovicidal activity. After 15 interaction days VC4 showed 21.8% of type 3 effect on parasitized eggs and VC1 showed 25.0% of type 3 effect. The authors also pointed out the need for field studies on the passage of *P. chlamydosporia* through the gastrointestinal tract of horses to assess the activity of this fungus against *O. equi* eggs in field conditions.

After 72 h of interaction, the isolate VC4 showed 28.4% of type 3 effect on *O. equi* eggs. This result, however, was recorded after the passage through the gastrointestinal tract of horses, indicating that *P. chlamydosporia* remained viable and

resistant under this condition and maintained its ovicidal activity on *O. equi* eggs in natural conditions.

De et al. (2008), investigated the predatory activity of *P. chlamydosporia* against *Fasciola gigantica* and *Gigantocotyle explanatum* and found that the fungus was effective in the destruction of their eggs in laboratory conditions after seven days of interaction. The authors reported that 2% CMA was the best culture medium for the fungus growth, which is interesting information, since according to Eren and Pramer (1965) the periodic supplying of nematodes to nematophagous fungi in nutritionally poor culture media would reduce their saprophyte growth. In this study, *P. chlamydosporia* germinated in plates containing faeces of treated animals using *O. equi* eggs as a nutritional source.

The results of this study demonstrated that the ovicidal activity of the isolate of *P. chlamydosporia* was kept throughout the experiment and destroyed *O. equi* eggs after 30 days of interaction.

O. equi eggs complete development in four to five days, containing the mature infective larval form (L_3) (Urquhart et al., 1998).

5. Conclusion

This is the first report on the viability and resistance of the nematophagous fungus *P. chlamydosporia* after passage through the gastrointestinal tract of horses without losing its predatory capacity against *O. equi*. The percentage results for the ovicidal activity obtained in this study allow us to suggest the use of *P. chlamydosporia* as an alternative biological control agent against eggs of this helminth, considering that its action will be concentrated on the faecal environment where *O. equi* eggs are found.

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Table 1 – Percentages of ovicidal activity for the nematophagous fungus *Pochonia chlamydosporia* (VC4) and the control, without fungal treatment, against eggs of *Oxyuris equi* at faeces collection times 8, 12, 24, 36, 48 and 72 hours, after 30 days of interaction

Isolate	Effect at 8 hours		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
VC4	18.8 ^{A}±7.0	22.4 ^{A}±8.9	29.1^{A}±11.3
Control	0^{B}±0	0^{B}±0	0^{B}±0
Isolate	Effect at 12 hours		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
VC4	20.8^{A}±8.1	23.9^{A}±8.8	28.2^{A}±11.6
Control	0^{B}±0	0^{B}±0	0^{B}±0
Isolate	Effect at 24 hours		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
VC4	21.4^{A}±8.8	24.2^{A}±9.3	31.1^{A}±14.5
Control	0^{B}±0	0^{B}±0	0^{B}±0
Isolate	Effect at 36 hours		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
VC4	22.9^{A}±7.8	26.5^{A}±9.6	27.4^{A}±10.9
Control	0^{B}±0	0^{B}±0	0^{B}±0
Isolate	Effect at 48 hours		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
VC4	22^{A}±11.1	23.4^{A}±9.2	30.9^{A}±12.6
Control	0^{B}±0	0^{B}±0	0^{B}±0
Isolate	Effect at 72 hours		
	Effect Type 1*	**Effect Type 2****	**Effect Type 3*****
VC4	21.5^{A}±11.2	26.1^{A}±9.3	28.4^{A}±12.4
Control	0^{B}±0	0^{B}±0	0^{B}±0

Percentages followed by same letter in the same column are not significantly different (P>0.01) – Friedman test. * Physiological, biochemical effect without morphological damage to eggshell, with hyphae adhered to the shell ** Lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell. *** Lytic effect with morphological alteration of embryo and eggshell, besides hyphal penetration and internal colonization

CAPÍTULO 4

Predatory activity of the nematophagous fungus *Duddingtonia flagrans* on horse cyathostomin infective larvae

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Abstract

This work was performed to determine the predatory capacity *in vitro* of the nematophagous fungus *Duddingtonia flagrans* (isolate AC001) on cyathostomin infective larvae of horse (L_3). The experimental assay was carried out on plates with 2% water-agar (2% WA). In the treated group, each plate contained 1.000 L_3 and 1.000 conidia of the fungus. The control group without fungus only contained 1.000 L_3 in the plates. Ten random fields (4mm diameter) were examined per plate of treated and control groups, every 24 hours for seven days under an optical microscope (10x and 40x Objective lens) for non-predated L_3 counts. After seven days, the non-predated L_3 were recovered from the Petri dishes using the Baermann method. The interaction there was a significant reduction ($p< 0.01$) of 93.64% in the cyathostomin L_3 recovered. The results showed that the *D. flagrans* is a potential candidate to the biological control of horse cyathostomin L_3.

Keywords: Nematophagous fungi - *Duddingtonia flagrans* - cyathostomin - Biological control – horse

1. Introduction

Raising livestock for commercial purposes leads to drastic losses mainly from parasitism by gastrointestinal helminths. The various damages caused by these infections are mainly related with decrease in yield, delayed growth rates, costs of veterinary treatment, therapy and, in some situations, death losses (Araújo et al. 2008). Most livestock in Brazil are still raised on pasture, leading to recurrent infections by parasites commonly found in these environments (Anualpec 2003). According to data from Brazilian Institute of Geography and Statistics, with 36 million horses, Brazil ranks third in the world in horse production. Strongyle nematodes are common in horses and an important group in Brazil, since great part of the herd is infected.

Current data based on field observations, parasitic load reduction and fecal egg counts suggest that horses become resistant to small strongyles with age. Nonetheless, this response is slow and inconsistent in most animals and has no relation with the intensity of previous contact with parasites (Anjos and Rodrigues 2006).

Worm control in horses is usually based on antihelminthics that have not been totally effective because their action restricted to adult parasites. Besides, the resistance to benzimidazole, the low possibility of new and more efficient chemical formulations, and the ecotoxicity of some compounds have attracted the interest for the development of new control practices to interfere with pasture infestation and that may contribute to lower anthelmintic use (Matthews et al. 2004).

Alternatives are required to help reduce the continued use of the same antihelmintic class. Biological control is among these alternatives, using natural nematode antagonistic fungi. The use of nematophagous fungi in the biological control of nematodiosis in domestic animal is a viable alternative (Braga et al. 2010). They are cosmopolitan species occurring in natural and agricultural soils and in all types of decomposing organic matter. Their action is concentrated in the fecal environment and aimed against free-living parasite larvae (Carvalho et al. 2009).

These organisms comprise different types of fungi divided into predators, endoparasites and opportunists, whose action is concentrated in the fecal environment and directed against free-living parasitic larvae. In the group of predators, the species *Duddingtonia flagrans* stands out as the most promising for the control of gastrintestinal nematodiasis in domestic animals (Braga et al. 2009a; Silva et al. 2009).

Predatory fungi are certainly the most studied groups and show the greatest potential for biological control (Araújo et al. 2008). The species *Duddingtonia flagrans*, *Monacrosporium thaumasium*, *M. sinense* and *Arthrobotrys robusta* are identified as

nematophagous fungi and have been studied for their potential as biological control agents for gastrointestinal nematodes of domestic animals (Braga et al. 2009b).

D. flagrans is considered the most promising nematode-trapping species in the control of nematodiosis in domestic animals due to its large chlamydospores production. This is the case of thick-walled spores that caused significant larval reduction in faecal cultures and pastures (Campos et al. 2008). It has predatory action by forming traps characterized by a system of simple adhesive hyphae that produces two types of spores - conidia and chlamydospores- interspersed by mature hyphae which in the environment can be used as a biological control agent (Larsen, 1999; Braga et al. 2007).

The present study evaluated the *in vitro* predatory capacity of the *D. flagrans* (isolate AC001) fungus on cyathostomin infective larvae.

2. Material and Methods

2.1. Fungi

An isolate of the *D. flagrans* (AC001) fungus was used in the experiment. The isolate was originally obtained from a soil located in Viçosa, Minas Gerais, Brazil, 20°45'20"S; 42°52'40" W, 649m altitude, using Duddington soil sprinkling method (1955) modified by Santos et al. (1991). The fungus was kept in test tubes containing 2% corn-meal-agar (2% CMA), in the dark, at 4^{o}C for 10 days.

2.2. Conidia collection

Culture disks (4mm in diameter) were removed from the fungal isolates kept in test tubes containing 2% CMA and transferred to 9.0cm Petri dishes containing 20mL of 2% potato dextrose agar and kept at 25°C in the dark for 10 days. After growth, new culture disks (4mm in diameter) were transferred to 9.0cm diameter Petri dishes containing 20mL of 2% water-agar (2% WA) and 1mL of distilled water containing 1000 larvae of *Panagrellus* sp. was added daily during 21 days to induce conidia formation. When fungal development was complete, 5mL of distilled water were added to each Petri dish, and the conidial and mycelial fragments were removed as described by Carvalho et al. (2009).

2.3. *Cyathostomin* larvae

Fresh feces were collected directly from the rectal ampulla of sixteen naturally infected mixed breed horses (*Equus caballus*) between 3-18 years old. The animals for the experiments were obtained from the Animal Science Department of the Federal

University of Viçosa, Viçosa - MG. Positive animals were found by the counting of eggs per gram of feces, according to Gordon and Whitlock (1939).

Coproculture was then established and after seven days L_3 were obtained, identified and quantified as described by Bevilaqua et al. (1993) under an optical microscope using a 10x objective lens. The Baermann technique showed that 100% of separated larvae were from cyathostomin. The recovered L_3 were counted in five aliquots of 10µl under an optical microscope using a 10x objective lens.

2.4. Experimental assay

Two groups (treated and control) were formed in 9.0cm plates containing 20mL of 2% WA, with 10 repetitions each. Petri dishes were previously divided into 4mm fields. In the treated group, each plate contained 1.000 L_3 and 1.000 conidia of the fungal isolate AC001. The control group (without fungus) contained 1000 L_3 larvae plated with 2% WA only.

Ten random fields (4mm diameter) were examined per plate of treated and control groups, every 24 hours for seven days under an optical microscope (10x and 40x Objective lens) for non-predated L_3 counts. After seven days, the non-predated L_3 were recovered from the Petri dishes using the Baermann method.

2.5. Statistical analysis

The mean of recovered cyathostomin L_3 was calculated. Data was examined by the analysis of variance at 1 and 5% probability (Ayres et al. 2003).

Predation efficiency of L_1 relative to the control group was evaluated by the Tukey's test at 1% probability. The reduction percentage of L_3 means was calculated according to the following equation:

$$\text{Reduction\%} = \frac{(\text{Average of } L_3 \text{ recovered from control} - \text{Average } L_3 \text{ recovered from treatment})}{\text{Average of } L_3 \text{ recovered from the control group}} \times 100$$

3. Results

The *D. flagrans* isolate AC001 was capable of predating L_3 larvae during the *in vitro* experiment. The predation was visualized in the plates of the treated group in the reading of the first field 24 hours after larvae and fungal isolate interaction. The presence of nematophagous fungi was not observed in the control group during the experiment. Evidence of predation was confirmed by the means of recovered cyathostomin L_3 using the Baermann method at 7 days post-plating, at the end of the experiment (Figure 1).

The mean of non-predated L_3 per 4 mm field during the experiment is shown in Table 1. At the end of seven days, the isolate AC001 showed better action comparing with the control and a significant reduction ($p<0.01$) of 93.64% in the mean number of L_3 recovered with the Baermann apparatus.

4. Discussion

D. flagrans is considered the most promising species in for biological control against gastrointestinal nematode parasites of livestock. It has been used successfully in several laboratory and field studies. *D. flagrans* has proven action against ruminant gastrointestinal nematodes and is a promising candidate for biological control of these organisms (Silva et al. 2009). The results of the present work showed that *D. flagrans* (AC001) had higher *in vitro* predatory activity on cyathostomin L_3.

Reduction in the number of L_3 per 4mm diameter field in the control group, during the study, was caused by larvae migration to the periphery of the Petri dishes, where moisture level was higher, which was also reported by Araújo et al. (2006) in an *in vitro* assay carried out in Petri dishes.

Eren & Pramer (1965) reported on the regular supply of nematodes to fungi in nutritionally poor culture media, which would reduce the fungal saprophytic growth. For this reason, in this study we used only 2% WA, a low nutrient culture medium, to reduce the saprophytic growth of fungal isolates providing cyathostomin L_3 as a single nutritional source. The higher mobility of nematodes can stimulate greater to trap production (Nansen et al. 1988). This phenomenon was observed in this study, as trap formation and L_3 predation by the fungal isolates were recorded during the first observation, 24 hours after interaction.

According to Fontenot et al. (2003), *D. flagrans* has an optimum growth rate, of 15 and 60 mm per week, at temperatures between 20 and 30°C. In the presence of nematodes, this fungus can produce 700 to 800 traps per cm^2 in short time. During the present work the temperature was kept around 28ºC, and there was an effective reduction in cyathostomin L_3 number because of the presence of *D. flagrans*. The large production of thick wall chlamydospores confers high capacity of passing through the gastrointestinal tract to *D. flagrans*, for which it is considered as a potential biological controller of gastrointestinal nematodes (Braga et al. 2009a). In the presence of trap structures produced by *D. flagrans*, L_3 and free-living nematodes are quickly killed before twenty-four hours of entrapment (Braga et al. 2009b).

In the present work, even with the administration of conidia, the fungus produced optimum results as early as the first 24 hours of reading (Table 1). Assis and Araújo (2003) working with *Monacrosporium sinense* and *M. appendiculatum* observed that at the end of fifteen days there was reduction of 70% of the mean number of recovered infective cyathostomin larvae from the plates and coprocultures, when compared with the control group ($p < 0.05$).

The results of the present work suggest that *D. flagrans* is more effective than the above species against cyathostomin infective larvae after seven days. *D. flagrans* is considered the most promising species in for biological control against gastrointestinal nematode parasites of livestock (Larsen 2006). It has been used successfully in several laboratory and field studies (Araújo et al. 2006). Baudena et al. (2000) proved the effectiveness of *D. flagrans* to reduce recovery of cyathostomin larvae from pastures, recording decrease in percentage of recovered larvae in the treated group compared with the control group, after receiving 2×10^6 spores per kilogram of live weight during 4 days.

The results showed that the fungal isolate AC001 was efficient and is a potential candidate to the biological control of horse cyathostomin infective larvae.

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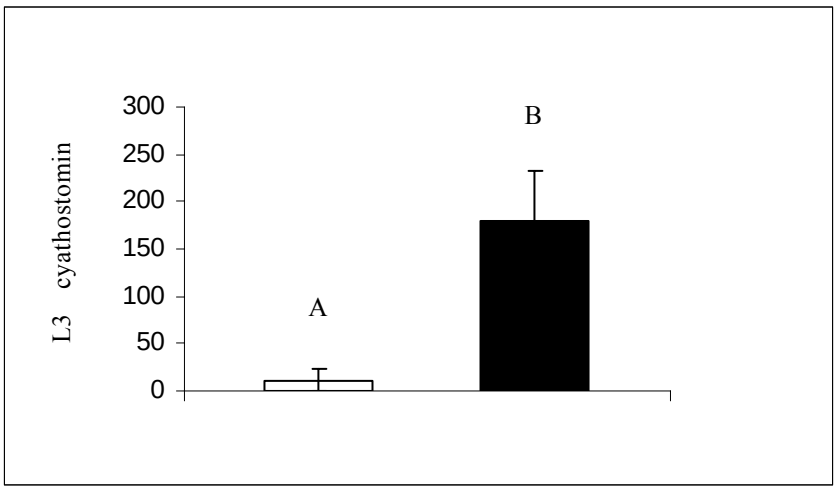

Fig.1 Mean number of non-predated cyathostomin infective larvae (L_3) recovered in 2% water-agar by the Baermann method at the seventh treatment day after interaction with the fungal isolate □*Duddingtonia flagrans* (AC001) and group control ■. Lines on Bars represent standard deviation. Means followed by at least one common capital letter (A) in the row are not significantly different by the Tukey's test at a 1% probability level.

Table 1 Daily average and standard deviations of cyathostomin infective larvae (L_3) non- predated for field of 4 mm of diameter in 2% water-agar during the period of 7 days in the treatments with the fungus *Duddingtonia flagrans* (AC001) and in the control without fungus

Time	Treatment (means of non-predated L_3)	
(days)	AC001	Control
1	1,8A ± 1,66	3,05B± 4,77
2	0,84A ± 0,80	2,09B ± 1,08
3	0,73A ± 0,92	2,26B ± 1,51
4	0,86A ± 1,08	2,51B ± 1,99
5	0,39A ± 0,85	2,29B ± 1,49
6	0,45A ± 0,83	2,96B ± 2,84
7	0,20A ± 0,38	2,97B ± 2,24

Means followed by the same capital letter are not significantly different ($p>0.01$).

CAPÍTULO 5

Ovicidal action of a crude enzymatic extract of the fungus *Pochonia chlamydosporia* against cyathostomin eggs

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Abstract

The aims of this study were to test the action of the fungal extract of *Pochonia chlamydosporia* (VC4) on the hatching of cyathostomin eggs plated in Petri dishes containing 2 % water-agar (2% WA) and its enzymatic activity in fecal cultures, in two experimental assays (A and B). The fungus *P. chlamydosporia* (VC4) was cultured in Erlenmeyer flasks (250 ml) containing 50 ml of liquid minimal medium supplemented with 0.2% gelatin for production of the crude enzymatic extract. Approximately 1 kg of fresh feces was collected directly from the rectum of crossbred horses naturally infected with cyathostomins. The fecal material was used to obtain eggs and prepare fecal cultures. For assay A, one thousand eggs were plated on 4.5 cm diameter Petri dishes together with 5 ml of VC4 fungal filtrate and incubated at 26 ºC in the dark for 24 hours. The control group consisted of 1000 eggs in Petri dishes containing 10 ml of distilled water, which were incubated under the same conditions. After 24 hours, the total number of cyathostomin larvae present in each plate of the treated and control groups was counted. For assay B, about 20 grams of feces were added with 10 ml of fungal extract of *P. chlamydosporia* (VC4) and incubated at 26 ºC for 8 days. Third stage larvae (L_3) were recovered at the end of this period. Significant difference ($p < 0.01$) was found for the number of larvae between the treated group and the control at end of assay A. A 72.8% reduction in the hatching of cyathostomin eggs was found in the plates of the treated group compared with the control group. At the end of 8 days, the fungal extract of *P. chlamydosporia* (VC4), in assay B, was effective in reducing the number of L_3 cyathostomins in the treated group by 67.0% compared with the control group. Significant difference ($p < 0.01$) was found between the means of L_3 recovered from the treated group and the control group. The results of this work showed that crude enzymatic extract of *P. chlamydosporia* (VC4) was effective in reducing hatching of cyathostomin eggs and therefore could be used as a biological control agent of this nematode.

Keywords: *Pochonia chlamydosporia*; fungal filtrate; horse; cyathostomin.

1. Introduction

Horses are hosts for a wide variety of helminths. Among them, the most important are nematodes, especially cyathostomins. Cyathostomin infections have been incriminated as a major cause of colic and diarrhoea in adult horses (Uhlinger, 1990; Love and Duncan, 1992; Murphy and Love, 1997). These parasites are the most prevalent in horses and larvae are present in the pasture throughout the year. However, the species distribution varies widely among different age groups (Barbosa et al., 2001; Quinelato et al., 2008).

Klei and Chapman (1999) reported field data suggesting that horses can acquire resistance to helminths with age, which is confirmed by the reduced parasite load and egg count in feces. This response is slow and inconsistent in most animals and unrelated to the intensity of previous contact with parasite.

Kaplan (2002) and Matthews et al. (2004) discussed that worm control in horses is usually carried out using anthelmintics. However, these drugs have not been totally effective in controlling nematodes since their action is restricted to adult forms and there is occurrence of resistance.

The continued use of the same antihelmintic class, the rapid rotation of compound groups, introduction of resistant worms and the use of doses lower than the recommendation should be avoided. Biological control using natural nematode antagonistic fungi is among the most viable alternatives. These organisms comprise different types of fungi whose action is concentrated in the fecal environment and directed against free-living parasitic larvae (Larsen, 1999; Thamsborg et al., 1999). Nematophagous fungi are the main natural antagonists of nematodes in the environment. These fungi are classified into predators, endoparasites and ovicidal. Among ovicidal fungi, the species *Pochonia chlamydosporia* and *Paecilomyces lilacinus* stand out as potential bio-nematicides (Carvalho et al., 2010). Nematophagous fungi secrete extracellular enzymes of the protease type that have an important role in the infection and destruction (ovicidal activity) of nematode eggs (Segers et al., 1994; Morton et al., 2004; Khan et al., 2004).

Some studies have shown that fungal isolates of *P. chlamydosporia* are capable of producing enzymes with activity against various types of solid substrates, such as polysaccharides, proteins and lipids (Esteves et al., 2009). Additionally, the ovicidal action of *P. chlamydosporia* (VC4) has been successfully tested against eggs of various genera of helminths in laboratory conditions (Braga et al., 2009a; 2010). Nonetheless, the crude enzymatic extract of this isolate has not been tested on eggs of gastrointestinal nematodes of domestic animals.

The objective of this study was to test the action of the fungal extract of *P. chlamydosporia* against cyathostomin eggs in two experimental assays (A and B).

2. Material and methods

2.1. Fungal culture

The isolate (VC4) from the ovicidal fungus *P. chlamydosporia* was obtained from the fungal collection of the Parasitology Laboratory at the Veterinary Department, Federal University of Viçosa, Minas Gerais, Brazil. Culture was carried out in test tubes containing 2% corn-meal-agar (2% CMA), at 4 °C, in the dark, for 10 days.

Culture disks (4 mm in diameter) of fungal isolates in 2% CMA were removed from the test tubes and transferred to 9.0 cm Petri dishes, containing 20 ml of 2% potato dextrose agar and kept at 25 °C in the dark for 10 days. After growth, new culture disks (4 mm in diameter) were transferred to 9.0 cm diameter Petri dishes containing 20 ml of 2% water-agar (2% WA) and kept at 26 °C in the dark for 10 days.

2.2. Production of crude extract of *P. chlamydosporia* (VC4) in liquid medium

Isolate (VC4) of *P. chlamydosporia* was cultured in Erlenmeyer flasks (250 ml) containing 50 mL of liquid minimal medium (0.3 g l/l NaCl, 0.3 g l/1 $MgSO_4.7H_2O$, 0.3 g l-1 K_2HPO_4 and 0.2 g l/1 yeast extract (Merck, Germany) and supplemented with 0.2% gelatin. Gelatin was filtered through Millipore filter (45 μm pore size) and aseptically added to the autoclaved medium. The samples containing the isolate were incubated in a shaker at 120 rpm at 28° C in the dark. After five days, the supernatant was collected and filtered in Whatman filter paper. no.1 at 4°C, according to Esteves et al. (2009).

2.3. Experimental assays

Two experimental *in vitro* assays, A and B, were performed in distinct steps during an interval of 8 days. In assay A, the effect of the crude enzyme extract of *P. chlamydosporia* (VC4) was evaluated on the hatching of plated cyathostomin eggs. In assay B, the enzymatic activity of crude extract of VC4 was evaluated on the hatching of cyathostomin eggs present in the fecal samples.

2.4. Fecal samples

Fresh feces was collected directly from the rectum of 8 crossbred horses (*Equus caballus*) naturally infected by cyathostomins. The animals were obtained from the horse experimental sector of Federal University of Viçosa, Viçosa, Minas Gerais, Brazil,

latitude 20°45’20” S, longitude 42°52’40” W. Feces samples were collected from all animals. The egg count per gram of feces (EPG) determined by the Gordon and Witlock (1939) method was performed to find positive animals. The mean EPG was 986.6. Positive EPG samples were homogenized and approximately 1 kg of fecal material was removed.

The fecal material was divided into two parts of approximately 500 g each. One part was used to recover eggs via the fecal flotation technique. These eggs were centrifuged in distilled water. The content of the centrifuge tube was homogenized and three 10 µl aliquots were removed. Eggs were counted under a stereomicroscope (10 x magnifications). The total number of eggs was estimated according to Araújo et al. (1993) and used in the experimental assay A. Cyathostomin eggs were evaluated for integrity under optical microscope (10 x magnification), according to Urquhart et al. (1998). The other part of the fecal material was used for coprocultures in assay B.

2.5. Assay A

A thousand cyathostomin eggs were plated in Petri dishes of 4.5 cm in diameter with 5 ml of crude extract of *P. chlamydosporia* (VC4), constituting the treated group. The control group consisted of 1000 eggs in 10ml of distilled in each plate without the fungal extract. The Petri dishes of treated and control groups were sealed with Rolapack film and incubated at 26 ºC in the dark for 24 hours. Each treatment consisted of six replications.

After 24 hours, the total number of cyathostomin larvae present in each Petri dish of the treated and control groups was determined according to the technique modified by Mukhtar and Pervaz (2003).

2.6. Assay B

Fresh feces with mean positive EPG count of 986.6 were used for preparing coprocultures, which were mixed with autoclaved and moistened vermiculite. Then, 10 ml of crude extract of *P. chlamydosporia* (VC4) were added to each coproculture, constituting the treated group. The control group consisted of coprocultures without the crude enzymatic extract of *P. chlamydosporia* (VC4). Both coprocultures, from treated and control groups, were incubated at 26 °C and for 8 days. At the end of this period, third stage larvae (L_3) were obtained by the Baermann method, which were identified and quantified according to the criteria described by Bevilaqua et al. (1993) under an optical

microscope using a ×10 objective lens. The Baermann reading showed that 100% of the viewed larvae were cyathostomins.

2.7. Statistical analysis

Data obtained in assays A and B were examined by analysis of variance at 1 and 5% probability levels using the BioEstat 3.0 software (Ayres et al., 2003). The efficiency of egg hatching in the control group was assessed by Tukey's test at 1% probability level. The percent reduction in the mean larval recovery was calculated by the following equation:

$$\text{Reduction\%} = \frac{[\text{Mean L3 recovered from control group} - \text{Mean L3 recovered from treated group}]}{\text{Mean L3 recovered from control group}}$$

3. Results

3.1. Assay A

After 24 hours of interaction, the presence of cyathostomin larvae and cyathostomin unhatched eggs with morphological alterations was observed in the plates of the group treated with the crude enzymatic extract of *P. chlamydosporia* (VC4), whereas, in the control group, only hatched larvae were found.

Data analysis showed significant difference ($p < 0.01$) in the number of larvae between the plates of the treated group and control group (Fig.1). Furthermore, there was 72.8 percent reduction in cyathostomin egg hatch in the plates of the treated group compared with the control group.

3.2. Assay B

At the end of 8 days, analysis of fecal cultures by the Baermann's technique showed that the fungal extract of *P. chlamydosporia* (VC4) was effective in reducing the number of cyathostomin L_3 (Fig. 2). Significant difference ($p < 0.01$) was found between the larval recovery rate in the treated group and the control group, with 67 percent reduction in the treated group.

4. Discussion

The present study showed the enzyme activity of the crude extract of *P. chlamydosporia* (VC4) on the hatching of cyathostomin eggs at the end of the experimental assay A. This result is in accordance with the findings of Esteves et al. (2009), who worked with *P. chlamydosporia* grown in liquid medium supplemented with

0.2% gelatin and subsequently demonstrated its action on egg hatching of the nematode *Meloidogyne* spp.

After 24 of incubation, it was observed that cyathostomin larvae were active, not showing paralysis. However, during the experimental assay, the toxicity of the crude extract of *P. chlamydosporia* (VC4) was not estimated by the number of paralyzed nematodes. On the other hand, Mukhtar and Pervaz (2003) reported that the action of the fungal filtrate of *P. chlamydosporia* on juveniles of the nematode *M. javanica* caused mortality of 13.25% of larvae after the same studied interval. The authors describe that the toxicity of the used fungal filtrate was estimated according to the mean percentage of paralyzed nematodes. The results found in this study, however, do not agree with those of Mukhtar and Pervaz (2003), probably because the different culture conditions and the use of gastrointestinal nematodes instead of phytonematodes.

Esteves et al. (2009) discussed that there may be differences in the results obtained in enzyme production among different isolates of the fungus *P. chlamydosporia*. Besides, these authors discuss that culture conditions seem to play an important role and should always be standardized so that meaningful comparisons can be made. Cayrol et al. (1989) argued that culture conditions can affect the toxic properties of a fungus. According to Weeb et al. (1972) and Segers et al. (1994) cited by Mukhtar and Pervaz (2003), the nematicidal action of the filtrate of *P. chlamydosporia* on juveniles of *M. javanica* can be attributed to the production of certain enzymes and toxins of the type verticillim A, B and C that help to weaken and dissolve the nematode cuticle. Khan et al. (2004) reported that nematophagous fungi produce enzymes that degrade different egg layers. However, there is no evidence of the action of *P. chlamydosporia* on infective larvae of gastrointestinal nematodes of domestic animals and further research is needed.

P. chlamydosporia produces a protease (VCP1) that is responsible for ovicidal activity against phytonematodes (Segers et al., 1994; Kerry and Hidalgo, 2004). According to Segers et al. (1996), production of VCP1 in liquid medium by the fungus *P. chlamydosporia* could be suppressed if the substrate gelatin were used in high concentrations. The results of this study showed that the fungus *P. chlamydosporia* (VC4) was able to grow in liquid medium supplemented with 0.2% gelatin and produce crude enzymatic extract with proteolytic action, however, the authors did not perform the enzyme purification and therefore it can not be affirmed that the proteolytic action was due to only the VCP1.

In the present study, the fungus *P. chlamydosporia* (VC4) was able to grow and produce crude proteolytic extract at five days of incubation. This result is comparable with

the findings by Esteves et al. (2009). In their work, the authors used a crude enzymatic extract of the fungus grown in the same time interval (5 days) that we used in our study. The authors tested three different day intervals (three, five and seven) and found that there was no difference ($p>0.05$) between the first two day intervals, whereas, at seven days of incubation, a decrease in protease production was recorded. Although there are reports in the literature on the action of filtrates from fungal cultures on the hatching and mortality of phytonematodes (Cayrol et al., 1989), this is the first report of the enzymatic activity of crude extract of this fungus on gastrointestinal nematodes of horses.

Several studies have mentioned the use of *P. chlamydosporia* (VC4) on eggs of helminth gastrointestinal parasites after 5 days of interaction (Araujo et al., 2009). Therefore, on the basis of the results of this work, the authors suggest that *P. chlamydosporia* could be used in the control of eggs of helminth gastrointestinal parasites that hatch in a short period of time in the environment. To date, studies on the ovicidal activity of the fungus *P. chlamydosporia* has only been reported on helminth eggs with longer period of incubation in the environment (Carvalho et al., 2010).

The fungus *P. chlamydosporia* (VC4) caused a 67% reduction in the number of L_3 recovered from the coprocultures at the end of assay B, demonstrating its enzymatic activity on eggs and possibly on some stages of the larvae, even in fecal material. In another study, Braga et al. (2010) showed that this isolate is able to survive the passage through the gastrointestinal tract of horses and maintain its ovicidal activity. Silva et al. (1999) reported the occurrence of small strongyles in horses from nine states in Brazil, identifying 23 species. These parasites are directly related to "larval cyasthostomiasis", a potentially fatal syndrome, and to the strong resistance of most gastrointestinal nematodes to routine antihelminths, showing the need of alternative control treatments. The infection in these animals occurs by ingestion of L_3 present in contaminated pastures (Reinemeyer, 1986, Reinemeyer and Herd, 1986; Braga et al., 2009b).

Braga et al. (2009b) reported that the predator fungus *Duddingtonia flagrans* caused decline in the percentage of cyathostomin L_3 recovery in both coprocultures and contaminated pastures with a 49.0 % reduction in L_3 recovered from coprocultures at the end of the experiment. The results obtained in assay B of this study allow the suggestion that crude enzymatic extract of *P. chlamydosporia* can be used as biological control agent against cyathostomin eggs. To our knowledge, this is the first report on the action of crude enzymatic extract of *P. chlamydosporia* against cyathostomin eggs. Thus, due to the importance of this biological control system, the authors suggest further studies on the characterization of the substances produced by the fungus *P. chlamydosporia* (VC4).

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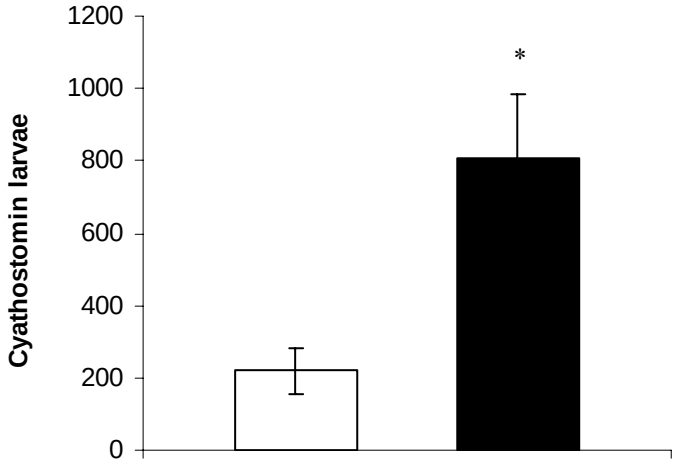

Fig. 1 Mean number and standard deviation (bar) of cyathostomin larvae recovered from plates of the treated group after 24 hours of interaction with the crude enzymatic extract of □*Pochonia chlamydosporia* (VC4) and in the control group ■. Asterisk denotes significant difference ($p<0.01$) between the fungus-treated group and the control - Tukey's test at a 1% probability level.

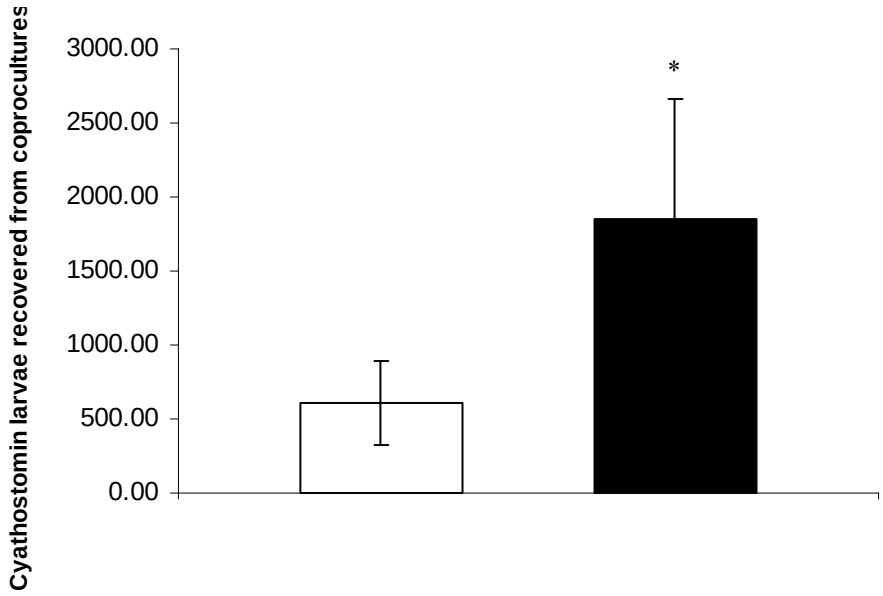

Fig. 2 Mean number and standard deviation (bar) of cyathostomin larvae recovered from coproculture of the group treated with the crude enzymatic extract of □*Pochonia chlamydosporia* (VC4) and the control ■, after 8 days. Asterisk denotes significant difference ($p < 0.01$) between the fungus-treated group and the control - Tukey's test at a 1% probability level.

CAPÍTULO 6

Optimizing protease production from an isolate of the nematophagous fungus *Duddingtonia flagrans* using response surface methodology and its larvicidal activity on horse cyathostomin

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Abstract

Protease production from *Duddingtonia flagrans* (isolate AC001) was optimized and the larvicidal activity of the enzymatic extract was evaluated on infective horse cyathostomin larvae (l_3). *D. flagrans* was grown in liquid medium with eight different variables: glucose; casein; bibasic potassium phosphate (K_2HPO_4), magnesium sulphate ($MgSO_4$), zinc sulfate ($ZnSO_4$), ferrous sulfate ($FeSO_4$) and copper sulfate ($CuSO_4$). The Placket-Burman analysis showed significant influence of $MgSO_4$, $CuSO_4$ and casein ($p<0.05$) on protease production by *D. flagrans* in liquid medium. The Central Composite Design indicated that the highest proteolytic activity was 39.56 U/ml as a function of the concentrations of casein (18.409g/l), $MgSO_4$ (0.10g/l) and $CuSO_4$ (0.50mg/l). Significant difference ($p<0.01$) was found for the larva number between the treated and control groups at the end of the experiment. It was found a reduction of 95.46% in the number of free-living larvae in the treated group compared with the control. The results of this study suggest that the protease production by *D. flagrans* (AC001) in liquid medium was optimized by $MgSO_4$, $CuSO_4$ and casein, showing that the optimized enzymatic extract exerted larvicidal activity on cyathostomin and therefore may contribute to a large-scale industrial production.

1. Introduction

Proteases are the most important group of commercial hydrolytic enzymes, constituting approximately 60% of the total enzymes produced worldwide (Rao *et al*., 1998) and proteases from microorganisms, such as nematophagous fungi, represent 40% of the total commercialized production (Godfrey & West, 1996; Meyer & Wiebe, 2003). Rao *et al*. (1998) reported that the production cost of these enzymes remains as one of the major obstacles to industrial application and that the high costs of the substrates used for microorganism cultivation are the main cause for its limited production. Thus, optimization of protease production by microorganisms can effectively contribute to cost reduction (Joo & Chang, 2005).

Nematophagous fungi capture and destroy nematodes, being natural antagonists of this parasite. Braga *et al.* (2009a) showed that these fungi can be successfully used in the control of gastrointestinal nematodes of horses including the Cyathostominae subfamily, which is prevalent in most of the Brazilian territory. *Duddingtonia flagrans* is considered the most promising species because of its large production of chlamydospores, resistant structures for survival. This species is also classified as predatory and can produce a range of enzymes, including proteases (Park *et al*., 2001; Meyer & Wiebe, 2003; Araújo *et al*., 2004).

In biotechnology research, the culture medium is usually optimized by a one-variable-at-a-time approach, in which all variables, except one, are kept constant, then the optimal level for the tested variable is determined. However, this method is too time consuming when there are a large number of variables (Kammoun *et al*., 2008).

According to Djekrif-Dakhmouche *et al*. (2006), statistical tools such as the Plackett–Burman design and the Response Surface Methodology (RSM) have been used for culture medium optimization, and Hajji *et al*. (2008) suggested that they can also be applied to investigate the interaction effect between the various parameters with the smallest possible number of experiments. RSM is an empirical statistical modeling technique to evaluate the relations between a group of controlled experimental factors and the observed results of a selected criterion (Ambati & Ayyanna, 2001). The factorial design and the regression analysis help to validate the effective factors and to build blocks for studying their interactions, serving as well to select the optimum conditions of the variables for a chosen response (Sharma *et al*., 2007).

In the present work, a response surface methodology was used to optimize the protease production by *Duddingtonia flagrans* and to evaluate its larvicidal activity.

2. Material and methods

Culture Conditions

The isolate (AC001) of the nematophagous fungus *D. flagrans* was obtained from a soil located in the Zona da Mata region, Viçosa, Minas Gerais, Brazil, 20°45'20"S, 42°52'40" W 20, 649 m altitude. The isolate was kept in test tubes containing corn-meal-agar 2% (2% CMA), at 4°C, in the dark for 10 days.

Fungal mycelia were obtained by transferring culture disks (approximately 5mm in diameter) of fungal isolates in 2% CMA into Erlenmeyer flasks (250 mL) containing 50mL of liquid medium according to the methodology modified by Meyer & Wiebe (2003). The medium consisted of glucose; casein; bibasic potassium phosphate (K_2HPO_4), magnesium sulphate ($MgSO_4$), zinc sulfate ($ZnSO_4$), ferrous sulfate ($FeSO_4$) and copper sulfate ($CuSO_4$) in varying concentrations as a function of the statistical treatment. Fungal inoculum was grown in rotary shaker at 120rpm and pH 9.0. After six days, the supernatant was collected and filtered in Whatman filter paper No. 1, at 4°C.

Enzymatic assay

The proteolytic activity was measured and modified as described by Joo & Chang (2005). Volumes of the solutions used in this method were: 100 mL crude extract, 400 mL Tris-HCl 100 mM, pH 7.0 and 500 μL of 1% casein pH 8.0. The reaction mixture was incubated at 43ºC for 15 minutes. The reaction was interrupted with 1 mL of 10% trichloroacetic acid. After 10 minutes, the medium was centrifuged at 10000 x g for 5 minutes, the supernatant was collected and absorbance determined in a 280 nm spectrophotometer. A standard tyrosine curve was constructed using various amounts of tyrosine. One protease unit was defined as the required enzyme quantity to release 1.0 μg of tyrosine per minute under the tested conditions.

Plackett-Burman design

The Plackett-Burman design is used for rapid multifactor screening to find the most significant physical and chemical independent factors required for high protease production (Plackett & Burman, 1946). Carbon and nitrogen sources previously tested by Meyer & Wiebe (2003) were added to the main culture medium for optimization. The Plackett-Burman design allows the validation of N variables in N +1 experiment. Each factor was tested at low (-1) and high (+1) levels. Table 1 shows the analyzed factors and respective levels and Table 2 the matrix of the Plackett-Burman experimental design with

the different levels (-1 and 1) of protease production. The Minitab Release 15 software was used to analyze the Plackett-Burman assays.

Central Composite Design

The Central Composite Design (CCD) is one of response surface methodologies (Chakravarti & Sahia, 2002). This design was used to determine the optimal concentration of the significant variables tested by the Plackett-Burman design. The effects of the significant variables on the enzymatic activity were assayed at 5 different levels:-α, -1, 0, 1, +α, where $\alpha = 2^{n/4}$, n is the number of variables and 0 corresponds to the central point. The experimental levels were selected using the Plackett-Burman design.

CCD with three factors and five levels, including six replicates at the central point was used to adjust the second order surface response. Protease production was analyzed by multiple regression using least squares to fit the following equation:

$$Y = a_0 + \sum a_i x_i + \sum a_{ii} x^2 + \sum a_{ij} x_i x_j$$

Where Y is the predicted response, a_o is the intercept, a_i is the linear coefficient, a_{ii} is the quadratic coefficient and aij is the interaction coefficient. xi and xj are the coded factor levels of variables Xi and X_j. Variable X_i was coded as x_i according to the following transformation equation:

$$x_{i=} (Xi\text{-}X_0)/\Delta Xi$$

Where x_i is the non-dimensional coded factor of X_i, $X0$ is the value of X_i at the central point, and ΔXi is the step point. The whole experiment was carried out in triplicate.

Larvicidal activity of the optimized enzymatic extract

Fresh feces were collected from the rectum of sixteen naturally infected, mixed breed horses (*Equus caballus*), 3-8 years old. Eggs per gram of feces (EPG) were counted as described by Gordon & Witlock (1939) to find positive animals. Coprocultures were then carried out and third stage larvae (L_3) were obtained after seven days, identified and quantified in an optical microscope (10 x objective) according to criteria proposed by Bevilaqua *et al*. (1993). Baermann readings showed that 100% of the detected L_3 were cyathostomin.

Treated and control groups were plated in Petri dishes (4.5 cm in diameter) with 6 replicates each. A total of 200 cyathostomin L_3 were incubated in Petri dishes containing 5 ml of the optimized enzymatic extract from *D. flagrans* (AC001). The plates were sealed with Rolapack film and incubated at 26°C in the dark for 24 hours. The control

group contained 200 of cyathostomin L_3 in distilled water (10ml) and incubated under the same conditions. The total number of cyathostomin L_3 was counted in each Petri dish of both groups after 24 hours (Mukhtar & Pervaz, 2003). Data was examined by analysis of variance (ANOVA) at significance levels of 1 and 5% probability. The Tukey test (1% probability level) was used to assess predatory efficiency of L_3 compared with the control (Ayres *et al*., 2003). Reduction percentage of mean larva number was calculated with the following equation:

$$\text{Reduction\%} = \frac{\text{Mean larvae of control} - \text{Mean larvae of treatment}}{\text{Mean larvae (control group)}} \times 100$$

3. Results and discussion

Selection of carbon and nitrogen sources

Preliminary studies by Meyer & Wiebe (2003) with glucose and casein as carbon and nitrogen sources for production of lytic enzymes by the same fungus were used as reference for this work. Nguyen *et al*. (2007) discussed that the growth of nematophagous fungi is directly linked to the carbon: nitrogen (C: N) ratio, while Araújo (2001) and Jaffee (2004) reported that the predatory capacity is enhanced by the presence of nematodes or substances derived from these parasites as a major nitrogen source. Glucose and casein were successfully used as carbon and nitrogen sources for the growth of *D. flagrans* (AC001) and protease production in this study.

Placket-Burman design

Eight variables were analyzed using the Plackett-Burman design with regard to their effects on protease production by *D. flagrans* (AC001) (Tables 1 and 2). Table 3 shows the results for the statistical analysis of the responses. Factors evidencing P-values of less than 0.05 were considered to have significant effect on the response (protease production). The selected factors $MgSO_4$, $CuSO_4$ and casein influenced ($p < 0.05$) the protease production of *D. flagrans* in liquid medium, whereas $ZnSO_4$ showed no effect ($p < 0.05$). Abidi *et al*. (2008) investigated the effect of divalent metal ions and cations in the production of fungal proteases and found that there was a higher proteolytic activity when the liquid medium was added with $FeSO_4$ and $CaCl_2$. In that study, however, the addition of $ZnSO_4$ and other salts had no effect on the production of proteases. This is partly in accordance with the results reported here, since $FeSO_4$ showed no significance ($p > 0.05$) in the production of proteases by *D. flagrans*.

Reports in the literature indicated that the ion Ca^{+2} is a good inducer of the enzymatic activity (Abidi *et al*., 2008), but this may not influence the protease production of specific fungal isolates. Since the ion Ca^{+2} was not tested in the present work, the authors used results obtained by Meyer & Wiebe (2003) who also evaluated the proteolytic production by *D. flagrans*, demonstrating that Ca^{+2} had no effect on the protease production.

Central Composite Design

Based on the results from the Plackett-Burman design, RSM was applied to determine optimal levels of the three selected variables ($MgSO_4$, $CuSO_4$ and casein). A total of 20 runs with different combinations of the selected factors were performed and the results for the full factorial experimental assay are shown in Table 4.

The function for the final response, which estimates the proteolytic activity after removing the non-significant variables ($p > 0.05$), is as follows:

$$\boldsymbol{Y = 16.5467 + 7.47339X_1 + 6.92795\ X_2 + 2.81501X_1^2 + 2.76552\ X_2^2 - 3.36510X_3^2 + 5.57875\ X_1\ X_2}$$

Where Y is the response value, which corresponds to the enzyme activity and X_1, X_2 and X_3 are the coded levels of $MgSO_4$, $CuSO_4$ and casein, respectively.

The statistical significance of the regression model was tested by the *F* test, and the analysis of variance (ANOVA) was used for the response surface quadratic model (Table 5).

A high R^2 indicates fitness of the model (Weisberg, 1985). The regression coefficients and analysis of variance in this work estimated R^2 as 0.93 (fit: 0.8707), showing the high significance of the model, which is consistent with the above statement. The results also indicated that the linear terms of $MgSO_4$ and $CuSO_4$ showed significant effect ($p < 0.001$) on the protease production by *D. flagrans*. Besides, the quadratic effect of $MgSO_4$, $CuSO_4$ and casein and the interactive effect between $MgSO_4$ and $CuSO_4$ also had significant effect, with the following values of $p > F$: (0022) $MgSO_4$; (0024) $CuSO_4$, (0009 and 0003) $MgSO_4$ and $CuSO_4$. On the other hand, the linear effect of casein, the interactive effect between $MgSO_4$ and casein and the interactive effect between $CuSO_4$ and casein showed no significance ($p > 0.05$) in this work.

Figure 1a-c presents the results for three-dimensional (3-D) response surfaces plotted as a function of the model equation for protease production by *D. flagrans* (AC001). The response surfaces were based on the final model, with one constant variable

fixed at its optimal level, while the remaining two factors varied within the experimental range.

Results of the effect of casein variation with either variable demonstrated that response surfaces for both combinations were similar (Fig.1a, b). The rise in the concentrations of $MgSO_4$ or $CuSO_4$ increased the protease production by *D. flagrans* to optimum values. However, Hajji *et al*. (2008) discussed that an excessive increase in substrate concentration could lead to a decrease in protease production by fungi.

An ascending curve shows the combined effect of $MgSO_4$ and $CuSO_4$, indicating a possible synergism between them (Fig. 1c). In addition, the authors of this work also suggest that the combination of these variables at higher levels could be significant for protease production by the *D. flagrans* (AC001) fungus.

Validation of the model

Validation was carried out under the conditions predicted by the model. The experimental values were very close to the predicted values; hence, the model was successfully validated. The validation of the statistical model and regression equation was performed by taking: casein (1.59 g/l), $MgSO_4$ (0.10 g/l) and $CuSO_4$ (0.50 mg/l) and casein (10.0 g/l), $MgSO_4$ (0.0159 g/l) and $CuSO_4$ (0.50 mg/l) in the experiment. The predicted response for protease production was, respectively: 13.59 U/ml and 16.22 U/ml, while the actual (experimental) response was, respectively: 12.82 U/ml and 16.93 U/ml, thus proving the validity.

Larvicidal activity of the fungal extract

The optimized enzymatic extract obtained from *D. flagrans* (AC001) exhibited larvicidal activity in the Petri dishes of the treated group after 24 hours of interaction (Fig. 2). No destruction of cyathostomin L_3 was observed in the control group in the same interval. There were differences ($p < 0.01$) between the number of L_3 in the treated group Petri dishes and the control group, with 95.4% percentage reduction of cyathostomin L_3 in the treated group in comparison with the control.

Proteases produced by nematophagous fungi have been extensively studied since they seem to take part in nematode cuticle degradation (Tunlid & Jansson, 1991). In this study, the optimized enzymatic extract of *D. flagrans* was effective in the destruction of cyathostomin L_3, suggesting proteolytic action. This is the first report on culture media optimization for protease production by *D. flagrans*.

According to Braga *et al*. (2009b), *D. flagrans* (AC001) has been successfully used in laboratory conditions in the biological control of gastrointestinal nematodes of humans and animals. In another study, Braga *et al*. (2009c) reported that the isolate AC001 of *D. flagrans* grown in 2%WA medium showed predatory activity against cyathostomin L_3 with percentage reduction of 97.5%, in laboratory conditions. The destruction of cyathostomin was confirmed with the use of optimized *D. flagrans* (AC001) enzymatic extract in this work.

Several studies have aimed at the improvement of the protease production by microorganisms (Gupta *et al*., 2002). The enzymatic production may be maximized since each fungal species has specific requirements in special conditions. As suggested by Araújo *et al*. (2004), the development of fungal formulations for biological control is a major step towards the commercial production of these microorganisms.

The authors suggest the characterization of substances produced by the fungus D. flagrans (AC001) in future works and indicate that this was the first step towards the use of enzymatic extract of *D. flagrans* as an alternative biological control of cyathostomin larvae, however, they admit the need for more studies in this area. Thus, studies aimed at producing fungal material in an economically viable manner are extremely necessary and an important step to enable the commercial production of nematophagous fungi and enzyme extract, including isolation, synthesis and applicability tests.

The results obtained in this study demonstrate that $MgSO_4$, $CuSO_4$ and casein optimized protease production by the fungus *D. flagrans* (AC001) in liquid medium and destroyed cyathostomin L_3.

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Table 1 High (+1) and low levels (-1) with the eight selected variables (g/l) : glucose (A), casein (B), K_2HPO_4 (C), $MgSO_4$ (D), $ZnSO_4$ (E), $FeSO_4$ (G), $CuSO_4$ (H) and temperature (ºC) in the Placket-Burman experimental design.

Table 2 Matrix of the Plackett-Burman experimental design with different production levels (-1 and 1) of proteases (U/ml) by the nematophagous fungus *Duddingtonia flagrans* (AC001).

Table 3 Factors (g/l): glucose, casein, K_2HPO_4, $MgSO_4$, $ZnSO_4$, $FeSO_4$, $CuSO_4$ and temperature (ºC) in the Plackett-Burman experimental design and significance ranking.

Table 4 Experiments used for response surface methodology (RSM) with three dependent variables (casein $MgSO_4$ and $CuSO_4$), with five levels each (-1, +1, 0, -1.68179 and 1.681793) and proteolytic activity values.

Table 5 Analysis of variance (ANOVA) for the response equation developed for protease production by *Duddingtonia flagrans* (AC001).

Table 1

Variables	Variable code	Low level (-1)	High level (+1)
Glucose	A	5	15
Casein	B	5	15
K_2HPO_4	C	2.5	7.5
$MgSO_4$	D	0.1	0.3
$ZnSO_4$	E	0.0025	0.0075
$FeSO_4$	F	0.0005	0.002
$CuSO_4$	G	0.000125	0.0005
Temperature	H	26	30

Table 2

Run	Variables								Act.
	Glucose	Casein	K_2HPO_4	$MgSO_4$	$ZnSO_4$	$FeSO_4$	$CuSO_4$	Temperature	(U/ml)
1	1	-1	1	-1	-1	-1	1	1	37.4795
2	1	1	-1	1	-1	-1	-1	1	0
3	-1	1	1	-1	1	-1	-1	-1	0.2192
4	1	-1	1	1	-1	1	-1	-1	0
5	1	1	-1	1	1	-1	1	-1	0
6	1	1	1	-1	1	1	-1	1	5.5342
7	-1	1	1	1	-1	1	1	-1	2.0822
8	-1	-1	1	1	1	-1	1	1	10.6849
9	-1	-1	-1	1	1	1	-1	1	0
10	1	-1	-1	-1	1	1	1	-1	13.1507
11	-1	1	-1	-1	-1	1	1	1	11.0137
12	-1	-1	-1	-1	-1	-1	-1	-1	11.726

Glucose: -1 (5g/l); 1 (15g/l); Casein: -1 (5g/l); 1 (15g/l); K_2HPO_4 : -1 (2.5g/l); 1 (7.5g/l); $MgSO_4$: -1 (0.1g/l); 1 (0.3g/l); $ZnSO_4$: -1 (0.0025mg/l); 1 (0.0075mg/l); $FeSO_4$: -1 (0.0005mg/l); 1 (0.002mg/l); $CuSO_4$: 1 (0.000125g/l); 1 (0.0005g/l): Temperature: 1 (26 ºC); 1 (30ºC).

Table 3

Constant	Effect	Coef	SE Coef	t-*test*	p>|*t*|	Ranking
		7.658	1.272	6.02	0.009	
Glucose	3.406	1.703	1.272	1.34	0.273	7
Casein	-9.032	-4.516	1.272	-3.55	0.038	3
K_2HPO_4	3.352	1.676	1.272	1.32	0.279	8
$MgSO_4$	-11.059	-5.530	1.272	-4.35	0.022	1
$ZnSO_4$	-5.452	-2.726	1.272	-2.14	0.122	5
$FeSO_4$	-4.721	-2.361	1.272	-1.86	0.161	6
$CuSO_4$	9.489	4.744	1.272	3.73	0.034	2
Temperature	6.256	3.128	1.272	2.46	0.091	4

Coef: coefficient; SE: standard desviation; t-*test:* is the value of the variables determined by Student's t-test at the 5% probability level p-value.

Table 4

Run Order	$MgSO_4$	$CuSO_4$	Casein	Activity (U/ml)
1	-1	-1	-1	3.89
2	1	-1	-1	13.42
3	-1	1	-1	7.23
4	1	1	-1	38.58
5	-1	-1	1	10.03
6	1	-1	1	9.59
7	-1	1	1	12.93
8	1	1	1	35.3
9	-1.68179	0	0	16.22
10	1.681793	0	0	39.56
11	0	-1.68179	0	16.6
12	0	1.681793	0	38.9
13	0	0	-1.68179	13.59
14	0	0	1.681793	7.23
15	0	0	0	19.67
16	0	0	0	15.87
17	0	0	0	14.52
18	0	0	0	15.73
19	0	0	0	15.07
20	0	0	0	17.26

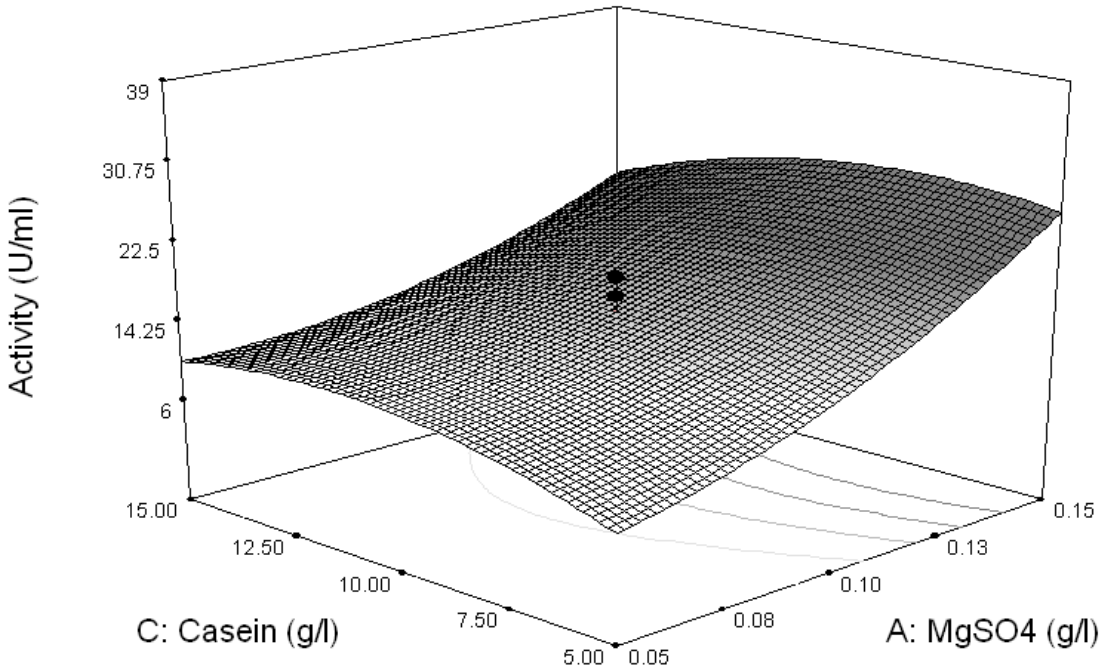
$MgSO_4$: -1 (0.05g/l); 1 (0.15g/l); 0 (0.10g/l); -1.68179 (0.015910g/l); 1.681793 (0.18409g/l). $CuSO_4$: -1 (0.25mg/l); 1 (0.75mg/l); 0 (0.50mg/l); -1.68179 (0.079552mg/l); 1.681793 (0.920448mg/l). Casein: -1 (5g/l); 1 (15g/l); 0 (10g/l); -1.68179 (1.5910g/l); 1.681793 (18.409g/l).

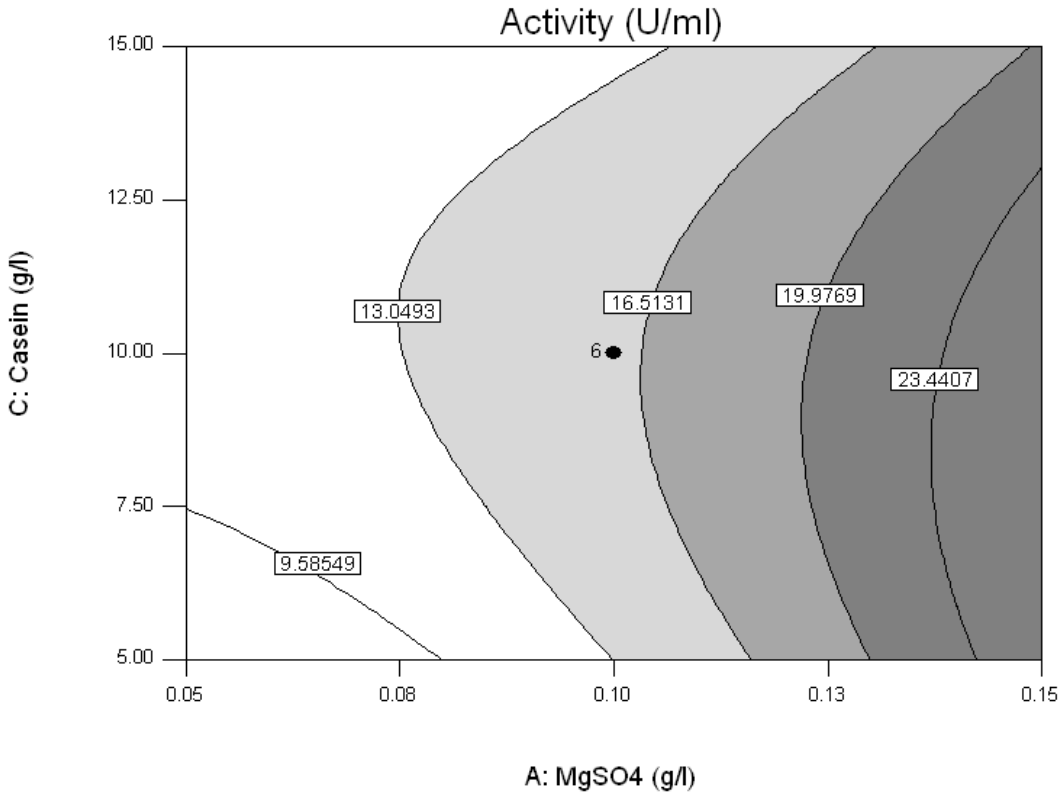
Table 5

Source	SS	DF	MS	*F*-value	*p* > *F*
$CuSO_4$					0.025
Casein					0.013
$MgSO_4$*$MgSO_4$					0.022
$CuSO_4$*$CuSO_4$					0.024
Casein*Casein					0.009
Model	2138.55	9	237.616	15.22	<0.001
Lack-of-Fit	138,70	5	27.741	7.95	0.020
Error	156.16	10	15.616		
Total	2294.71	19			

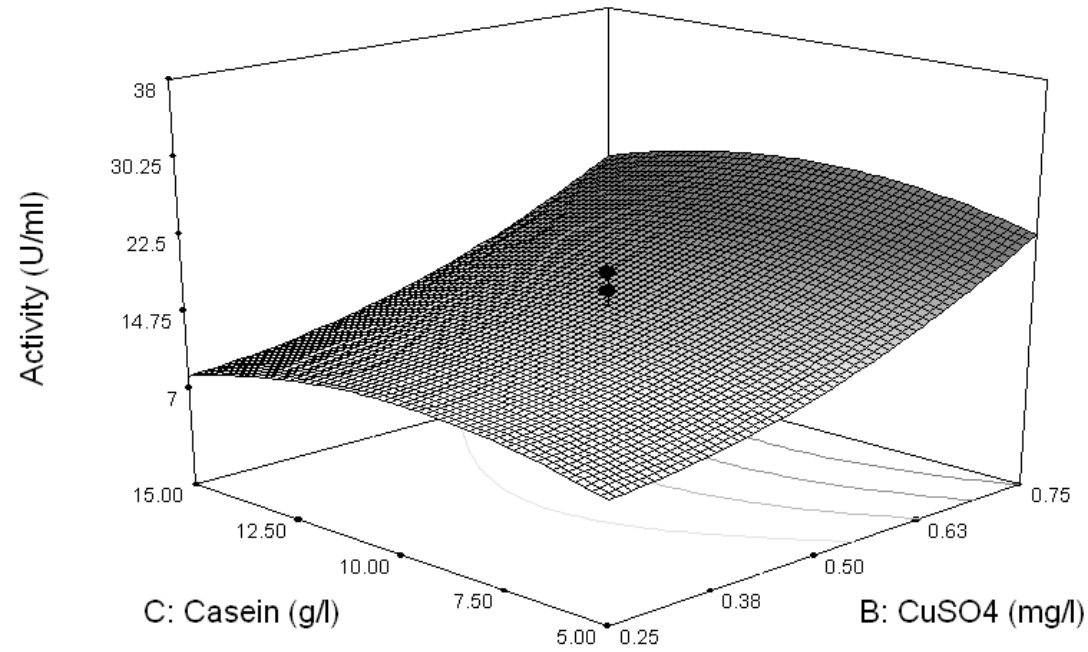
R^2: 0. 9319 R^2 (adj): 0.8707. SS= Sum of Squares; DF=Degrees of Freedom; MS=Mean of Squares.

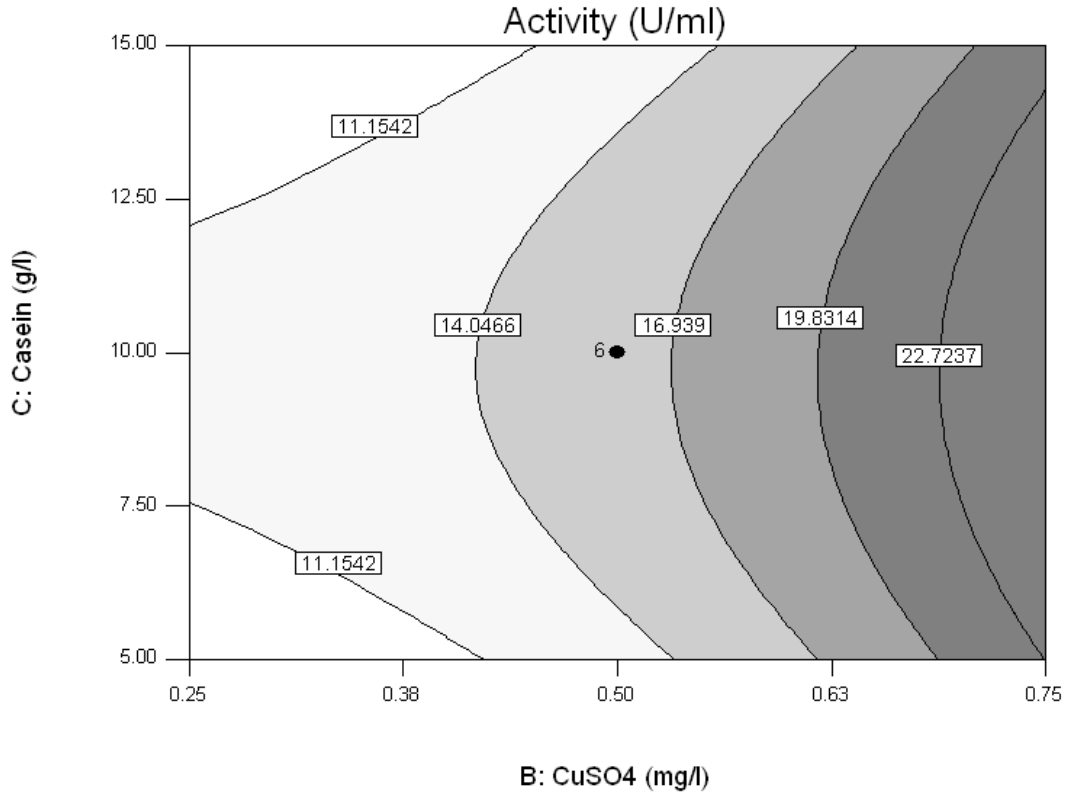
a)


Activity (U/ml)
39
30.75
22.5
14.25
6
15.00
12.50
10.00
7.50
5.00
C: Casein (g/l)
0.05
0.08
0.10
0.13
0.15
A: MgSO4 (g/l)


Activity (U/ml)
15.00
12.50
10.00
7.50
5.00
C: Casein (g/l)
13.0493
16.5131
19.9769
23.4407
9.58549
6
0.05
0.08
0.10
0.13
0.15
A: MgSO4 (g/l)

b)


Activity (U/ml)
38
30.25
22.5
14.75
7
15.00
12.50
10.00
7.50
5.00
C: Casein (g/l)
0.25
0.38
0.50
0.63
0.75
B: CuSO4 (mg/l)


Activity (U/ml)
15.00
12.50
10.00
7.50
5.00
C: Casein (g/l)
11.1542
14.0466
16.939
19.8314
22.7237
6
11.1542
0.25
0.38
0.50
0.63
0.75
B: CuSO4 (mg/l)

c)

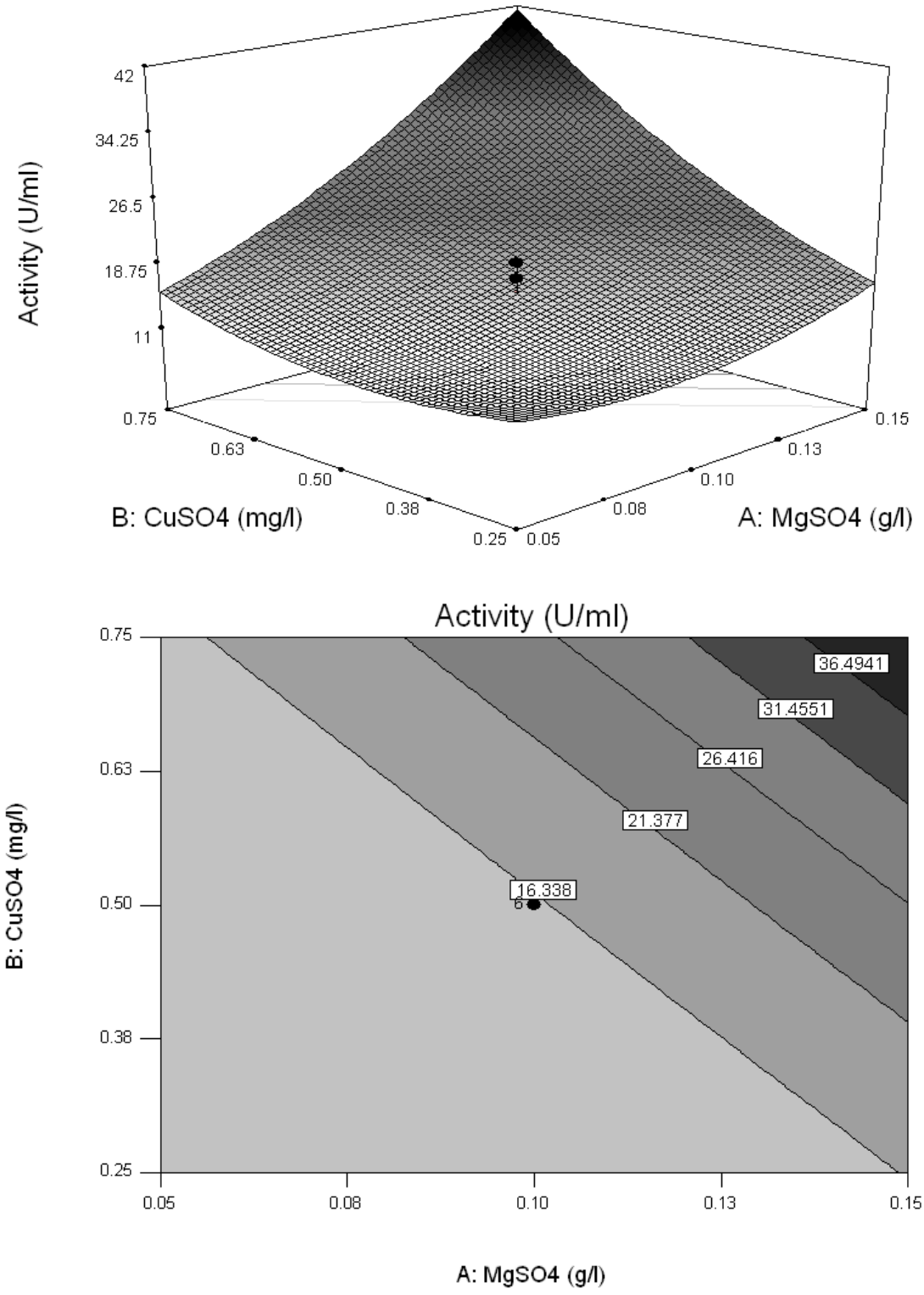


Fig. 1 Response surface curve (RSM) and contour map of the protease production by *Duddingtonia flagrans* (AC001) and the interaction between the variables $MgSO_4$ and casein (a); $CuSO_4$ and casein (b); $MgSO_4$ and $CuSO_4$ (c).

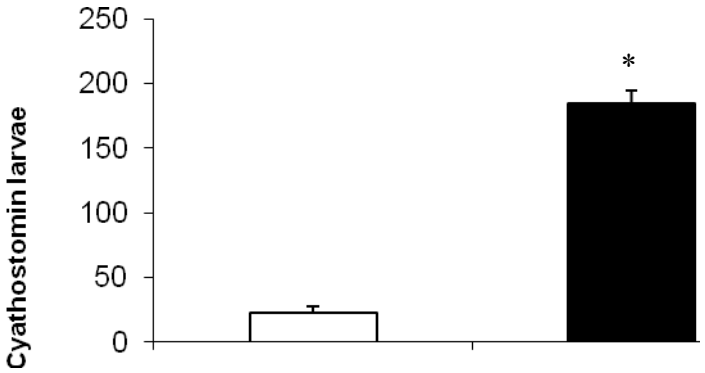


Fig. 2 Mean number and standard deviation of L_3 cyathostomes recovered after 24 hours of interaction in Petri dishes with optimized enzyme extract of □*Duddingtonia flagrans* (AC001) and control group ■. Significant difference ($p < 0.01$) between treated group and control (*) – Tukey's test.

CAPÍTULO 7

Analysis of growth, enzymatic production and *in vitro* ovicidal effect of *Pochonia chlamydosporia* and *Paecilomyces lilacinus* on *Oxyuris equi* eggs

Abstract

The aim of this work was to analyze the growth, enzymatic production and ovicidal effect of *Pochonia chlamydosporia* (VC1 and VC4) and *Paecilomyces lilacinus* (PL1) on *Oxyuris equi* eggs. The growth of isolates (VC1, VC4 and PL1) and their enzymatic production were evaluated on agar media supplemented with gelatin (GA), casein (CA), olive oil (OOA) or starch (SSA). The ovicidal effect was evaluated according to the following parameters: type 1 effect, physiological and biochemical effect without morphological damage to eggshell with hyphae adhered to the shell; type 2, lytic effect with morphological damage to the eggshell and type 3, lytic effect with egg destruction. Following, the *P. chlamydosporia* crude extract production and proteolytic content was evaluated (VC1 and VC4) in liquid medium during incubation intervals of 15, 30, 45 and 60 minutes. *P. chlamydosporia* and *P. lilacinus* grew and showed enzymatic activity on agar media (GA, CA, OOA and SSA). At the 15th day , VC1, VC4 and PL1 showed results for type 3 effect of (77.6%, 54.0%, 51.8% and 67.8%), (72.4%, 50.0%, 58.4% and 77.6%) and (62.4%, 57.5%, 65.3% and 63.5%), on GA, CA, OOA and SSA media, respectively. *P. chlamydosporia* was able to grow in liquid medium (gelatin) and at day five showed proteolytic activity Linear growth of proteolytic activity for VC1 and VC4 was observed in the intervals. The results of the present work suggest that *P. chlamydosporia* and *P. lilacinus* can be used in the biological control of *O. equi* eggs.

Keywords: Nematophagous fungi, *Pochonia chlamydosporia*, *Paecilomyces lilacinus*, proteases, *Oxyuris equi*.

1. Introduction

Eggshells and cuticles of gastrointestinal nematodes are infection sites for microorganisms used in the biological control of phytonematodes and gastrointestinal nematodes of domestic animals (Huang et al., 2004; Araújo et al., 2008). Proteases, (VCP1) chitinases and other hydrolytic enzymes are usually produced and secreted by ovicidal nematophagous fungi to damage nematode eggshell and cuticle (Segers et al., 1996; Gortari et al., 2008).

Among the nematophagous fungi, the species *Pochonia chlamydosporia* and *Paecilomyces lilacinus* stand out for their capacity to secrete extracellular enzymes (Araújo et al., 2008; Braga et al., 2008), such as proteases (Morton et al. 2004; Segers et al., 1994; Khan et al., 2004), which play an important role in nematode egg infection. Some studies have shown that *P. chlamydosporia* and *P. lilacinus* isolates may produce enzymatic extracts against various types of solid substrates, including proteins, lipids and polysaccharides (Gortari et al., 2008; Esteves et al., 2009).

The ovicidal action of *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) has been successfully tested against eggs of several helminths under laboratory conditions (Carvalho et al., 2010; Silva et al., 2010). These isolates, however, have never been assayed for their proteolytic potential, neither on solid medium against gastrointestinal nematode eggs of domestic animals nor as fungal crude enzymatic extract.

Oxyuris equi is a gastrointestinal nematode known to parasite horses and to produce harmful effects by the feeding habits of third and fourth stage infective larvae (Bowman et al., 2006). Pregnant female parasites instead of releasing their eggs in the fecal bulk migrate to the lower part of the colon and rectum and out of the anus to deposit egg masses (80 to 60 thousand eggs) on the anal skin and the perianal region, causing anal itching (Urquhart et al., 1998). Anthelminthics are used in the control of worm parasites in horses, but they may not be completely effective mainly because they can lead to parasite resistance (Bowman et al., 2006; Matthews et al., 2004).

Growth, enzymatic production and ovicidal effect of *P. chlamydosporia* and *P. lilacinus* grown on solid media against *O. equi* eggs were assessed in this work. The proteolytic potential of *P. chlamydosporia* (VC1 and VC4) crude extract was also tested.

2. Material and methods

2.1. Fungus species

Three nematophagous fungal isolates, (VC1 and VC4) from *P. chlamydosporia* and (PL1) from *P. lilacinus*, were kept in test tubes containing 2% corn-meal-agar (2% CMA), at 4° C in the dark for 10 days. These isolates were obtained from the mycological collection of the Laboratory of Parasitology, Department of Veterinary Medicine, Federal University of Viçosa, Minas Gerais, Brazil.

Culture disks (4 mm in diameter) were extracted from the fungal cultures kept in test tubes containing 2% CMA, transferred to 9-cm Petri dishes containing 20 mL of 2% potato-dextrose agar (2% PDA) and kept at 26º C in the dark for 10 days. After fungal growth, new 4-mm culture disks were transferred to 9-cm Petri dishes containing 20 mL of 2% water-agar (2%WA) and kept at 26° C in the dark for 10 days.

2.2. Obtaining *Oxyuris equi* eggs

Eggs of *O. equi* were recovered by dissection of adult female specimens from feces of an infected horse and identified according to Urquhart et al. (1998). The eggs were washed 10 times in distilled water and centrifuged at 1,000 rpm for 5 minutes each time. The supernatant was discarded at the end of each centrifugation cycle. The eggs were then incubated at 25ºC for 14 days with a solution containing 0.05% formalin, 0.005% streptomycin sulphate and 0.01% chloramphenicol as described by Araújo et al. (1995). The work was divided into two experimental assays, A and B, carried out in different stages during 15 days. Assay A assessed growth, enzymatic production and ovicidal effect of *P. chlamydosporia* (VC1 and VC4) *and P. lilacinus* (PL1) on agar

media with different supplementation against eggs of *O. equi*. Assay B evaluated the crude extract production in liquid medium supplemented with gelatin 0.2%, the proteolytic activity in different incubation periods and the protein content of *P. chlamydosporia* (VC1 and VC4).

2.3. Assay A

2.4. Growth and enzymatic production of *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) on solid medium

Growth and enzymatic production of *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) were studied on different agar medium substrates: gelatin (GA), casein (CA), olive oil (OOA) and starch (SSA) and 2% WA. Water- agar (2%WA) was used as control medium. The following enzymatic activities were tested for each isolate: Proteolytic Activity (Konema and Roberts, 1985; Kunert et al., 1987) in gelatin agar (GA) and casein agar (CA); Lipase Activity (Kunert and Lysek, 1987) in olive oil agar (OOA) and Amylolytic Activity (Carrillo and Molina, 1998) in starch agar (SSA).

Petri dishes (9.0 cm in diameter) containing the different supplemented agar media (GA, CA, OOA and SSA) were inoculated with 4-mm diameter culture disks of *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) and incubated at 26º C in the dark for 10 days. Enzymatic activities were determined according to the corresponding substrate degradation in colonies with an average diameter of 5 cm, regardless of incubation time (Kunert et al. 1987; Gortari et al., 2008), with 10 repetitions for each treatment.

The results for each supplemented agar medium (GA, CA, OOA and SSA) were interpreted by considering the ratio between the degradation halo radio of substrate and the colony according to the following scale: grade 0, no degradation; grade 1, degradation occurring in the colony centre; grade 2, degradation occurring in the whole colony; grade

3, degradation radio 2-10 mm larger than that of the colony; grade 4, degradation radio 10 mm larger than that of the colony (Kunert et al., 1987).

2.5. Ovicidal effect of *P. chlamydosporia* and *P. lilacinus* on eggs of *O. equi*

Eggs of *O. equi* were morphologically analyzed for their integrity under light microscopy (10x objective lens) and placed on 9.0 cm Petri dishes with agar media supplemented with gelatin (GA), casein agar (CA), Olive oil agar (OOA) and starch agar (SSA) with fungal isolates grown for 10 days and control without fungus, at 26ºC, with ten replicates for each group. Each Petri dish contained one thousand eggs of *O. equi* with one fungal isolate and a control without fungi. At 10 and 15 days, a hundred eggs were removed from each plate containing the fungal isolates and from the control (without fungi) as described by Araújo et al. (1995). The eggs were placed on glass slides with a drop of 1% Amam blue and evaluated under light microscopy (40x objective lens) according to Lysek et al. (1982): type 1 - physiological and biochemical effect with no morphological damage to eggshell and with hyphae adhered to the shell; type 2 - lytic effect with morphological alteration of the eggshell and embryo without hyphal penetration; and type 3 - lytic effect with morphological alteration of eggshell and embryo with hyphal penetration and internal egg colonization. Data from assay A were examined by the Friedman test at 1% probability level (Ayres et al., 2003).

2.6. Assay B

Crude extract production of *P. chlamydosporia* (VC1 and VC4) in liquid medium

Two *P. chlamydosporia* isolates (VC1 and VC4) were cultured in Erlenmeyer flasks containing 50 mL of liquid minimal medium (0.3 g/l NaCl, 0.3 g/1 $MgSO_4.7H_2O$, 0.3 g/l K_2HPO_4 and 0.2 g/1 yeast extract (Merck, Germany), supplemented with gelatin 0.2%. Gelatin was filtered through a 45μm Millipore filter before it was added aseptically into autoclaved medium**.** The samples were incubated at 28° C on a rotary shaker (120 x g) in

the dark. After five days, the supernatant was collected and filtered using filter paper **(**Whatman N° 1), at 4 ° C (Esteves et al., 2009).

2.7. Proteolytic activity at different incubation time and protein content of the crude extract of *P. chlamydosporia* (VC1 and VC4)

The proteolytic activity of VC1 and VC4 was measured using the modified caseinolytic method by Joo and Chang (2005). Volumes of used solutions were: 100 µl of filtered crude extract, 400µl of buffer Tris-HCl 100 Mm (pH 7.0) and 500µl of 1% casein pH 8.0 (CRQ-Chromate Chemical LTDA, Diadema Sao Paulo, Brazil). The reaction medium was incubated at 15, 30, 45 and 60 minutes, at 43° C. The reaction was stopped by adding 1 ml of 10% Trichloroacetic Acid (TCA). After 10 minutes, the reaction medium was centrifuged (5 min at 10.000g) and the supernatant was collected to calculate absorbance on a spectrophotometer (PG Instruments Ltd) at 280 nn. A tyrosine standard curve was built to quantify enzyme activity. One protease unit was defined as the amount of enzyme needed to produce 1.0µ m of tyrosine per minute (pH 7.0), at 43° C. The following factors were calculated to estimate the optimal incubation time for activity: activity (U/ml), protein content (µg/ml), total activity (U), total protein content (µg) and specific activity (U/µg). Protein content was determined according to Bradford (1976) and a standard curve for protein content quantification was built using bovine serum albumin (BSA-InLab).

3. Results

Assay A

Growth and enzymatic production of *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) on solid medium

Table 1 shows qualitative and semi-quantitative results for the assessed enzymatic activities of *P. chlamydosporia* and *P. lilacinus*. Positive enzyme activities for VC1, VC4

and PL1 were found on all the tested media. The descriptive analysis of Kunert et al. (1987) showed no difference between *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) on the culture media (GA and CA) for proteolytic activity in this study. Likewise, no difference was found between VC1, VC4 and PL1 on the OOA medium for the lipase activity. Amylase activity showed no difference among the three tested isolates on SSA medium.

Ovicidal effect of *P. chlamydosporia* and *P. lilacinus* against eggs of *O. equi*

Tables 2 and 3 show the results of the ovicidal activity percentage of *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) on *O. equi* eggs at 10 and 15 interaction days. All three isolates (VC1, VC4 and PL1) showed percentage results for effects type 1, 2 and 3 against eggs of *O. equi* on the four tested solid agar media (GA, CA, OOA and SSA).

The results of the ovicidal activity percentage for isolates were: VC1 (40.8%, 53.6%, 49.5%, 70.9% and 77.6%, 54.0%, 51.8%, 67.8%) respectively, on agar media GA, CA, OOA and SSA at 10 and 15 interaction days; VC4 (43.2%, 48.2%, 58.4% , 73.9% and 72.4%, 50.0%, 58.4%, 77.6%) respectively, on agar media GA, CA, OOA and SSA at 10 and 15 interaction days; PL1 (54.8%, 57.8%, 63.4%, 54.3% and 62.4%, 57.5%, 65.3%, 63.5%) respectively, on agar media GA, CA, OOA and SSA at 10 and 15 interaction days.

No difference ($p>0.01$) was found for the ovicidal effect (effect type 3) against eggs of *O. equi* between isolates VC1 and VC4 on gelatin (GA), casein (CA) and olive oil (OOA) agar media, at day 10. However, on starch agar medium (SSA), there was difference ($P <0.01$) in the action against eggs between these isolates. Difference ($p <0.01$) was found in the ovicidal activity of isolate PL1 compared with the isolates VC1 and VC4 on the media GA, OOA and SSA.

At day 15, difference ($p<0.01$) was found for type 3 effect between VC1 and VC4 only in gelatin agar. There was also a difference ($p<0.01$) for the ovicidal action of PL1 in relation to isolates VC1 and VC4, on the media GA, OOA and SSA.

The highest percentage results for effect type 3 were observed on gelatin (GA) and starch (SSA) agar media for all three isolates (VC1, VC4 and PL1) at the end of the experiment. Linear regression curves for type 3 effect also showed that isolates VC1, VC4 and PL1 had increased ovicidal activity against eggs of *O. equi* in GA medium: 36.8, 29.2 and 25.2, respectively (Fig. 1).

Assay B

Proteolytic activity at different incubation times and protein content of *P. chlamydosporia* (VC1 and VC4) crude extract

Figure 2 shows the proteolytic activity of VC1 and VC4 extracts at different incubation times. During the tested intervals (15, 30, 45 and 60 minutes) both isolates showed a linear increase of the proteolytic activity. Proteolytic activity of (0 and 0.49 U/ml) was found at 15 minutes; (0.53 and 6.66 U/ml) at 30 minutes; (3.4, 7.42 U/ml) at 45 minutes and (6.13 and 8.17 U/ml) at 60 minutes, for the isolates VC1 and VC4, respectively. The highest values for proteolytic activity of both VC1 and VC4 were found at 60 minutes.

The following values were obtained for the different factors at 60 minutes: (6.1369 and 8.1712) Activity (U/ml); (101.21 and 44.716) Protein Content (g/ml); (613.70 and 817.12) Total Activity (U); (10121.12 and 4471.6) Total Protein Content and (6.0635 and 0.18270) Specific activity (U/μg), for the isolates VC1 and VC4, respectively.

4. Discussion

Assay A

Growth and enzymatic production of *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) on solid medium

All fungal isolates showed growth and ovicidal activity against eggs of *O. equi* in the experimental assay A. Positive enzyme activity (proteolytic, lipolytic and amylolytic) were detected on all supplemented agar media for isolates VC1, VC4 and PL1. These results are consistent with the work of Gortari et al. (2008) and Esteves et al. (2009).

The mechanism of penetration of the ovicidal fungi into parasitized eggs is still not fully explained. However**,** several authors admit that enzymatic activity **(**proteases and chitinases) is a major component in the attack and penetration of eggs, (Lysek et al., 1982). Esteves et al. (2009) reported proteolytic activity of *P. chlamydosporia* when grown in liquid medium supplemented with 0.2% gelatin, which was also obtained in this work. However, this is the first report of the proteolytic activity of *P. chlamydosporia* on solid agar media supplemented with gelatin (GA) and casein (CA).

The chitinolytic activity in agar for the fungus *P. lilacinus* was not tested in this study. However, Gortari et al. (2008) did not detect any chitinolytic activity of *P. lilacinus* grown in chitin supplemented agar.

The proteolytic activity of *P. lilacinus* on agar medium supplemented with gelatin (GA) and casein (CA) was demonstrated in the present work. The results are consistent with the work of Gortari et al. (2008), who found high proteolytic activity for this fungus grown on agar media supplemented with the same substrates.

Nevertheless, environmental adaptability and the production of certain extracellular enzymes involved in the infective process are two of the main characteristics for the selection of a fungus species as potential biological control agent (Olivares-Bernabeu and López-Llorca, 2002). Still, according to Gortari et al. (2008), a pre-selection of fungi with ovicidal potential against these eggs could be based on the production of

proteases and chitinases. Therefore, the results in this study show that both *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* (PL1) could be used as biological control agents.

Ovicidal effect of *P. chlamydosporia* and *P. lilacinus* on *O. equi* eggs

Assay A showed that isolates (VC1 and VC4) and PL1 were effective over 15 days and were capable of destroying *O. equi* eggs and, thus, could be used as an alternative biological control of this helminth.

Using *P. chlamydosporia* (isolates VC1 and VC4) against eggs of *O. equi* in an *in vitro* assay, Braga et al. (2009) reported that both isolates showed predatory activity against this nematode at 5, 10 and 15 days, with no significant difference ($p > 0.01$) between their ovicidal activity (effect type 3). VC1 and VC4 showed percentage results of 25.0% and 21.8%, respectively at 15 days of interaction. These authors used only 2% WA medium for growth and subsequent analysis of ovicidal activity against eggs of *O. equi*. In the present work, the action of *P. chlamydosporia* (VC1 and VC4) was tested on agar medium supplemented with four different substrates, and it was found that the ovicidal activity was higher after 15 days for all tested substrates (Tables 1 and 2). Gelatin agar (GA) also showed increased ovicidal activity between 10 and 15 days, suggesting that the isolates can produce high protease concentrations (Fig.1). The higher nutrient percentages on the supplemented agar media may explain the difference in the percentage results for effect type 3 in the present study. On the other hand, Gortari et al. (2008) reported that the high proteolytic activity detected for *P. lilacinus* on medium supplemented with gelatin (GA) and casein (CA) might be related to the action of proteases found in this fungus species.

Assay B

Production of crude extract of the fungus *P. chlamydosporia* (VC1 and VC4) in liquid medium

The proteolytic activity tested in the GA medium showed higher ovicidal percentages against eggs of *O. equi* (effect type 3) at the end of the experimental period (Fig.1). This result is in agreement with the findings of Esteves et al. (2009), who worked with *P. chlamydosporia* in liquid medium supplemented with 0.2% gelatin.

P. chlamydosporia (VCP1) produces a type of protease that destroys eggs of phytonematodes and nematodes (Kerry and Hidalgo, 2004, Araújo et al., 2008). According to Segers et al. (1996) the production of VCP1 by *P. chlamydosporia* in liquid medium could be suppressed if gelatin medium in high concentrations were used. Assay B showed that *P. chlamydosporia* (VC1 and VC4) grew in 0.2% gelatin liquid medium with proteolytic activity. However, the authors' objective was not enzyme purification, but the production of crude enzymatic extract with proteolytic activity.

In the present work the 0.2% concentration of the substrate gelatin was used following the procedure described by Esteves et al. (2009). *P. chlamydosporia* (VC1 and VC4) was the only fungus species used in the assay, due to the following reasons: 1) the isolates VC1 and VC4 showed higher percentages of ovicidal activity (effect type 3) against eggs of *O. equi* than *P. lilacinus* (PL1) at the end of assay A; 2) the search for more information on the proteolytic activity of these isolates, since no difference in their action has been found ($p>0.01$) by several other works; 3) one seeks to study all relevant characteristics of isolates VC1 and VC4, as they have been proven to be potential biological control agents.

The fungus *P. chlamydosporia* (VC1 and VC4) was capable to grow and to produce proteolytic crude extract at five days of incubation. Esteves et al. (2009) used crude enzymatic extract of *P. chlamydosporia* also grown in the same time interval. These

authors tested three different intervals (3, 5 and 7 days) and found no difference ($p>0.05$) between days 3 and 5, but at day 7, there was a decrease in protease production.

Proteolytic activity at different incubation times and protein content of the crude extract of *P. chlamydosporia* (VC1 and VC4)

The proteolytic activity at different incubation times for VC1 and VC4 showed that the best results of the proteolytic activity occurred at the interval of 60 minutes, which is also consistent with the work of Esteves et al. (2009), who used the same incubation interval for *P. chlamydosporia*.

In the present work, we tested increasing times for the incubation of *P. chlamydosporia* extracts to obtain the optimum time for proteolytic activity. Esteves et al. (2009) suggested that from 60 min, stabilization of *P. chlamydosporia* proteolytic activity might take place, which was confirmed in our work. This is, however, the first report on the time of the proteolytic activity of *P. chlamydosporia* (VC1 and VC4). Besides, this is important information, as a number of studies have mentioned the action of the isolates VC1 and VC4 against eggs of gastrointestinal helminth parasites starting from five days of interaction (Araújo et al. 2008; Braga et al., 2009). The results indicate that *P. chlamydosporia* can be used in the control of eggs of gastrointestinal helminth parasites that hatch in a short period of time in the environment, because, so far, only helminth eggs with long periods of development in the environment have been studied (Braga et al ., 2008).

Both isolates VC1 and VC4 were effective regarding the factors studied for the interval of 60 minutes: total activity, total protein content, and specific activity. Nonetheless, the specific activity (proteolytic enzyme production) recorded for isolated VC1 was higher than for isolated VC4. We suggest that although both isolates were equally effective in egg destruction, the isolate VC1 stands out for a higher protease

production. Yet, further studies on the specific activity of VC1 and VC4 are needed, since this was the first report on this subject.

Gray (1983) discussed that knowledge on the factors that affect the natural control of nematodes is important for the use of biological control agents under laboratory and natural conditions. The understanding of these factors is crucial because the selection of a possible biological control agent must be based on its adaptive ability in the environment to which it is intended to be used, and not only under the isolation of laboratory conditions.

In the present work, we report the growth and enzymatic activity of *P. chlamydosporia* (VC1 and VC4) and *P. lilacinus* with destruction of *O. equi* eggs and, therefore, they could be considered in further experiments searching for their possible use as biological control agents against this parasite.

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Table 1 Enzymatic activity of *Pochonia chlamydosporia* (VC1 and VC4) and *Paecilomyces lilacinus* (PL1) on solid agar medium supplemented with gelatin (GA), casein (CA), olive oil (OOA) and starch (SSA) independent of incubation time.

Activity tested	Solid media	*Pochonia chlamydosporia*		*Paecilomyces lilacinus*
		VC1	**VC4**	**PL1**
Proteolytic	GA	4	4	3
	CA	4	4	3
Lypolytic	OOA	2	3	3
Polysaccharolytic	SSA	4	4	4

Grade 0, no degradation; Grade 1, degradation occurring in the colony centre; Grade 2, degradation occurring in the whole colony; Grade 3, degradation radio 2-10 mm larger than that of the colony; Grade 4, degradation radio 10 mm larger than that of the colony.

GA: gelatine agar. CA: casein agar. OOA: olive oil agar. SSA: mineral salts-starch agar.

Table 2 - Percentages of ovicidal activity and standard deviations of *Pochonia chlamydosporia* (VC1 and VC4) and *Paecilomyces lilacinus* (PL1) on solid agar medium supplemented with gelatin (GA) casein (CA), olive oil (OOA) and starch (SSA) and control without fungi against eggs of *Oxyuris equi* at 10 days of interaction.

Effect type 1* at 10 days				
Isolates	**Solid media**			
	GA	**CA**	**OOA**	**SSA**
VC1	32.0^{A}±13.2	11.9^{A} ±7.4	16.4^{A} ±6.9	10.0^{A} ±7.1
VC4	34.7^{A} ±7.8	20.3^{A} ±13.4	10.2^{A} ±4.2	7.8^{A} ±5.9
PL1	18.0^{B} ±7.7	16.8^{A} ±12.2	7.5^{B}±7.4	13.5^{A} ±4.3
Control	0^{C} ±0	0^{B}±0	0^{C}±0	0^{B}±0
Effect type 2 at 10 days**				
Isolates	**Solid media**			
	GA	**CA**	**OOA**	**SSA**
VC1	27.2^{A}±6.0	34.5^{A} ±8.0	32.1^{A} ±5.3	19.1^{A} ±5.2
VC4	23.1^{A}±11.4	30.5^{A} ±9.6	31.9^{A} ±6.7	18.2^{A} ±6.2
PL1	26.1^{A}±6.9	25.4^{B}±4.8	29.1^{A} ±9.8	30.6^{B}±4.9
Control	0^{B}±0	0^{C}±0	0^{B}±0	0^{C}±0
Effect type 3* at 10 days**				
Isolates	**Solid media**			
	GA	**CA**	**OOA**	**SSA**
VC1	40.8^{A} ±7.2	53.6^{A}±11.0	49.5^{A} ±7.0	70.9^{A} ±9.6
VC4	43.2^{A} ±11.6	48.2^{A}±13.8	57.4^{A} ±7.9	73.9^{A}±8.3
PL1	54.8^{B}±8.5	57.8^{A}±13.9	63.4^{B}±10.4	54.3^{B} ±6.2
Control	0^{C} ±0	0^{B}±0	0^{C} ±0	0^{B}±0

*Physiological, biochemical effect without morphological damage to eggshell, with hyphae adhered to the shell. **Lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell. ***Lytic effect with morphological alteration of embryo and eggshell, besides hyphal penetration and internal colonization.

GA: gelatine agar. CA: casein agar. OOA: olive oil agar. SSA: mineral salts-starch agar.

Table 3 – Percentages of ovicidal activity and standard deviations of *Pochonia chlamydosporia* (VC1 and VC4) and *Paecilomyces lilacinus* (PL1) on solid agar medium supplemented with gelatin (GA) casein (CA), olive oil (OOA) and starch (SSA) and control without fungi against eggs of *Oxyuris equi* at 15 days of interaction.

Effect type 1* at 15 days				
Isolates	**Solid media**			
	GA	**CA**	**OOA**	**SSA**
VC1	7.6^{A}±4.8	12.7^{A} ±6.1	18.0^{A} ±4.6	7.3^{A} ±7.4
VC4	7.1^{A} ±7.8	18.3^{A} ±13.4	10.0^{B}±6.2	8.3^{A} ±4.7
PL1	12.5^{A} ±4.9	17.9^{A} ±11.9	11.4^{B}±7.9	8.0^{A} ±8.2
Control	0^{B}±0	0^{B}±0	0^{C}±0	0^{B}±0
Effect type 2 at 15 days**				
Isolates	**Solid media**			
	GA	**CA**	**OOA**	**SSA**
VC1	30.3^{A}±9.2	18.6^{A} ±12.1	30.2^{A} ±5.0	25.9^{A} ±7.0
VC4	31.1^{A}±11.4	20.5^{A} ±9.6	32.0^{A} ±5.9	18.6^{A} ±9.1
PL1	24.5^{A}±6.7	25.1^{A}±8.7	28.0^{A} ±10.4	29.5^{BA}±7.3
Control	0^{B}±0	0^{B}±0	0^{B}±0	0^{C}±0
Effect type 3* at 15 days**				
Isolates	**Solid media**			
	GA	**CA**	**OOA**	**SSA**
VC1	77.6^{A} ±4.7	54.0^{A}±11.0	51.8^{A} ±7.9	67.8^{A} ±13.3
VC4	72.4^{B}±14.2	50.0^{A}±14.0	58.4^{A} ±7.9	77.6^{A}±4.8
PL1	62.4^{B}±9.1	57.5^{A}±8.0	65.3^{BA}±11.0	63.5^{AB} ±11.3
Control	0^{C} ±0	0^{B}±0	0^{C} ±0	0±0

*Physiological, biochemical effect without morphological damage to eggshell, with hyphae adhered to the shell. **Lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell. ***Lytic effect with morphological alteration of embryo and eggshell, besides hyphal penetration and internal colonization.

GA: gelatine agar. CA: casein agar. OOA: olive oil agar. SSA: mineral salts-starch agar.

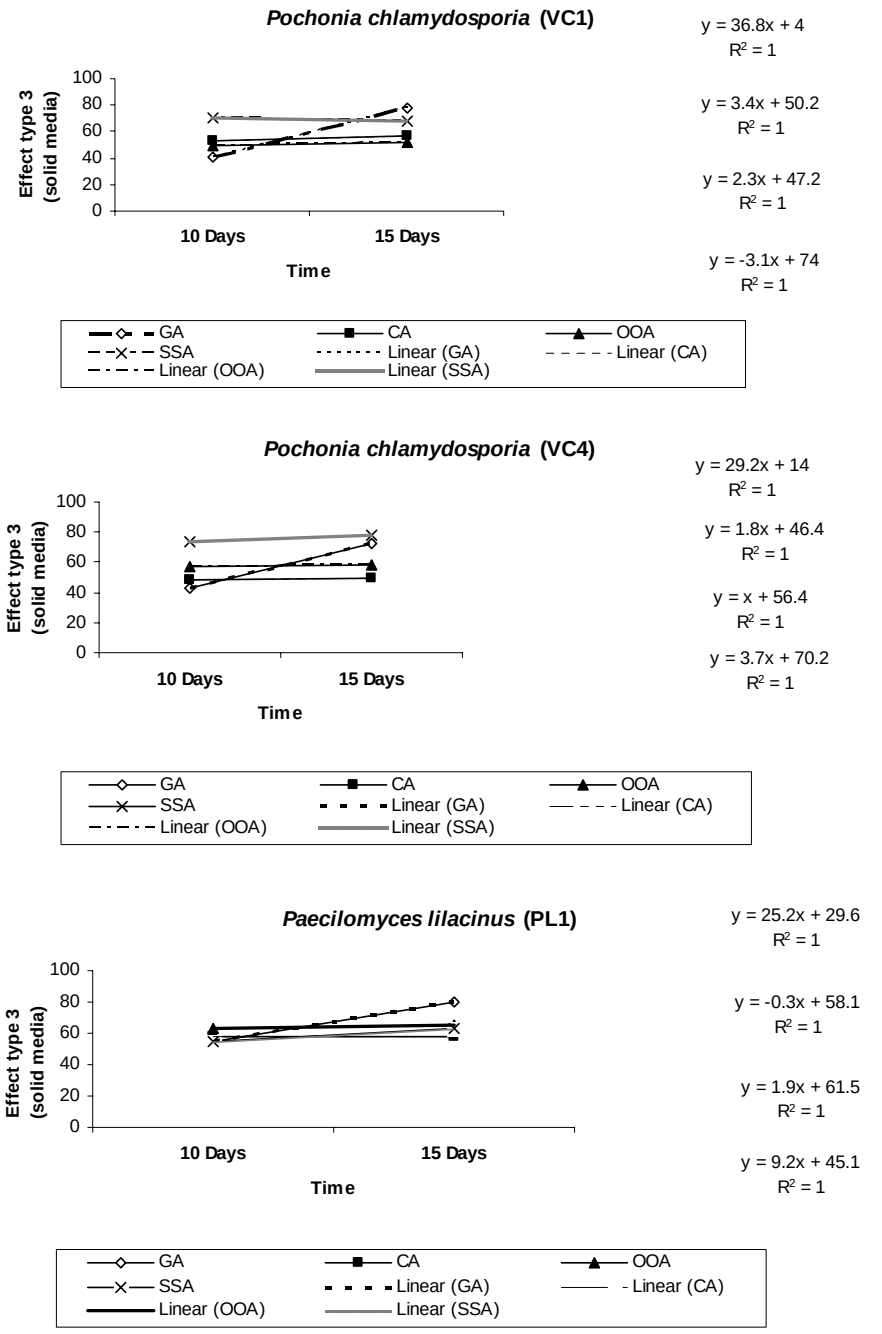

Fig.1 Linear regression curves for effect type 3 on solid agar media supplemented with gelatin (GA) casein (CA), olive oil (OOA) and starch (SSA) against eggs of *Oxyuris equi* at 10 and 15 days of interaction.

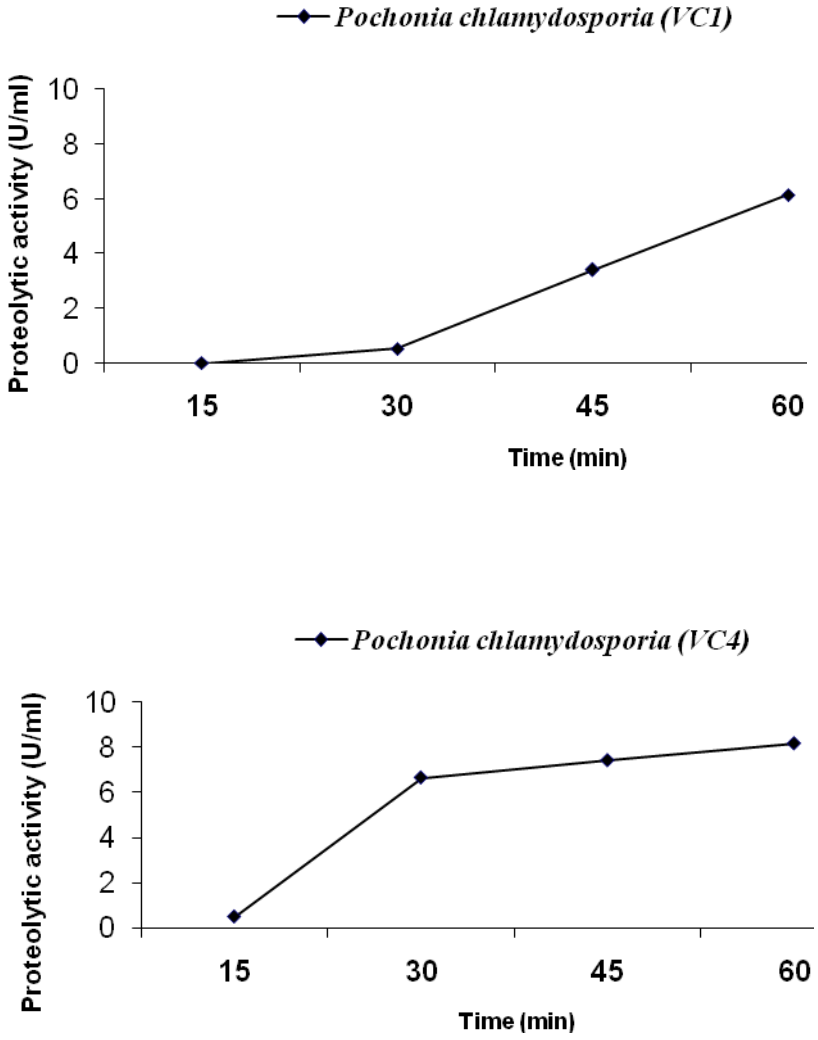

Fig. 2 – Proteolytic activity of the fungal extracts of *Pochonia chlamydosporia* (VC1 and VC4) at different incubation times (15, 30, 45 and 60 minutes).

3. CONCLUSÕES GERAIS

Pode-se concluir que:

1. O tratamento de equinos com péletes contendo massa miceliana do fungo nematófago *Duddingtonia flagrans* (AC001) foi efetivo no controle de ciatostomíneos em região tropical do sudeste do Brasil. Entretanto, seria interessante realizar um tratamento anti-helmíntico prévio para provocar a queda da carga parasitária presente nos animais e, por conseguinte no OPG e a partir disso fornecer o fungo na alimentação dos animais para controlar as formas larvais presentes no meio ambiente prevenindo, com isso, a reinfecção.

2. O isolado do fungo *Duddingtonia flagrans* (AC001) foi eficiente na redução *in vitro* de larvas infectantes de ciatostomíneos, confirmando os resultados de trabalhos anteriores. Dessa, *D. flagrans* pode ser utilizado no controle biológico de ciatostomíneos em condições laboratoriais.

3. O sulfato de magnésio ($MgSO_4$), o sulfato de cobre ($CuSO_4$) e a caseína otimizaram a produção de proteases pelo fungo *Duddingtonia flagrans* (AC001) em meio líquido causando a destruição de L_3 de ciatostomíneos. Sendo assim, este fato poderia contribuir para pesquisas futuras que visem a produção industrial em larga escala desse fungo.

4. O fungo *Pochonia chlamydosporia* (VC1 e VC4) influenciou de forma negativa os ovos de *Oxyuris equi* (ensaio A) e *Austroxyuris finlaysoni* (ensaio B) e assim pode ser considerado como um potencial candidato a controlador biológico desses nematóides. Todavia, o mecanismo de penetração dos fungos ovicidas nos ovos parasitados ainda não está totalmente elucidado e trabalhos futuros sobre que visem a interação desses isolados fúngicos e distintos ovos de helmintos são importantes.

5. O fungo *Pochonia chlamydosporia* (VC4) resistiu à passagem pelo aparelho gastrintestinal de equinos sem perder sua capacidade predatória sobre ovos de *Oxyuris equi* ao final do ensaio experimental. Dessa forma, por meio dos resultados apresentados sugere-se que o fungo *P. chlamydosporia* poderia ser

utilizado como uma ferramenta no controle biológico de ovos de *O. equi* em condições laboratorias e a campo.

6. O extrato bruto enzimático produzido de *Pochonia chlamydosporia* (VC4) foi eficaz na redução da eclosão dos ovos de ciatostomíneos e, sendo assim poderia ser utilizado no controle biológico desse nematóide. Todavia, no futuro, maiores estudos sobre as atividades enzimáticas dessa substância devem ser o foco de outros trabalhos.

7. Os fungos *Pochonia chlamydosporia* (VC1 e VC4) e *Paecilomyces lilacinus* cresceram em meios ágar suplementados com gelatina (GA), caseína (CA), óleo de oliva (OOA) e amido (SSA). Além disso, produziram atividade enzimática e destruíram os ovos de *O. equi* e, sendo assim podem ser empregados no controle biológico de ovos de *O. equi*. Todavia, maiores estudos sobre as atividades hidrolíticas dos fungos *P. chlamydosporia* e *P. lilacinus* devem ser o foco de outros trabalhos, com a finalidade de contribuir para a determinação dos principais eventos sobre a interação desses fungos sobre os ovos de helmintos parasitos gastrintestinais.