ISABELA DE CASTRO OLIVEIRA

ASSOCIAÇÃO DOS FUNGOS *Monacrosporium sinense* E *Pochonia chlamydosporia* NO CONTROLE BIOLÓGICO DE NEMATOIDES GASTRINTESTINAIS DE BOVINOS

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*.

Orientador: Jackson Victor de Araújo

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Assentimento:

Isabela de Castro Oliveira
Autora

Jackson Victor de Araújo
Orientador

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"Tenham coragem. Não tenham medo de sonhar coisas grandes".

(Papa Francisco)

RESUMO

OLIVEIRA, Isabela de Castro, D.Sc., Universidade Federal de Viçosa, julho de 2021. **Associação dos fungos *Monacrosporium sinense* e *Pochonia chlamydosporia* no controle biológico de nematoides gastrintestinais de bovinos**. Orientador: Jackson Victor de Araújo.

As infecções por nematoides são uma importante restrição à produção eficiente de ruminantes criados em pastagem no Brasil e no mundo. O presente estudo descreve sobre os fungos *Monacrosporium sinense* (SF53) e *Pochonia chlamydosporia* (VC4) e seu uso associado no controle de nematoides gastrintestinais de bovinos. As etapas dos estudos *in vitro* envolveram observação dos índices de velocidade de crescimento micelial (IVCM) de cada fungo em três meios de cultura, seis temperaturas e sete variações de pH; a compatibilidade entre estes isolados; microscopia eletrônica e óptica de suas estruturas e interações com nematoides e suas atividades nematicidas conjuntas e isoladas sobre larvas infectantes (L3) de bovinos. A etapa do estudo *in vivo* avaliou os efeitos da administração oral de péletes contendo a combinação fúngica no controle biológico de L3 de nematoides parasitas gastrintestinais de bovinos criados em pastagens. A compatibilidade entre os isolados SF53 e VC4 foi confirmada pelos testes de confrontação direta, antibiose e compostos voláteis. Além disso, a avaliação nematicida *in vitro* mostrou que a melhor eficácia foi quando os dois isolados foram usados em conjunto, com redução de 98,90% no número de L3 de nematoides de bovinos. A espécie *P. chlamydosporia* apresentou a maior taxa de crescimento em meio ágar água a 20°C, enquanto *M. sinense* apresentou um crescimento numericamente melhor a 30°C. Os fungos não cresceram a 35 ou 40°C durante os seis dias de observação. Surpreendentemente, o crescimento micelial de ambos os isolados inibidos na temperatura de 35°C por 6 dias, recomeçaram seu crescimento quando a temperatura foi reduzida para 25°C. Já a temperatura de 40°C foi prejudicial para o desenvolvimento de ambos os fungos no laboratório. A observação do pH foi importante para mostrar que as variações de pH no trato gastrointestinal dos bovinos não serão prejudiciais aos fungos, uma vez que oferecer formulações orais aos animais é a forma mais prática de dispersar fungos nas fezes. A microscopia eletrônica e óptica mostrou as estruturas fúngicas que beneficiam seu uso no controle biológico de nematoides e interações com larvas infectantes de helmintos. Larvas de nematoides mantidas pelo micélio de *P. chlamydosporia* confirmam sua capacidade de predar os estágios larvais, apesar de ser um fungo classificado no grupo "ovicida". Na avaliação a campo, a recuperação de L3 da pastagem do grupo tratado com a combinação fúngica foi 91,7% e 86,4% menor do

que na pastagem do grupo controle, respectivamente para amostras coletadas a 20 cm e 40 cm das fezes. Os valores de ovos por grama de fezes (OPG) nos meses de maio de 2019 e janeiro de 2020 foram menores ($p \leq 0.05$) no grupo tratado. Portanto, os fungos *M. sinense* e *P. chlamydosporia* são promissores no controle de larvas infectantes de nematoides gastrintestinais de bovinos.

Palavras-chave: Agentes biocontroladores. Compatibilidade. Crescimento micelial. Fungos nematófagos. Helmintos. Parasitas. Pastagem. Predadores. Ruminantes. Verminoses.

ABSTRACT

OLIVEIRA, Isabela de Castro, D.Sc., Universidade Federal de Viçosa, July, 2021. **Association of the fungi *Monacrosporium sinense* and *Pochonia chlamydosporia* in the biological control of bovine gastrointestinal nematodes**. Advisor: Jackson Victor de Araújo.

Nematode infections are an important restriction to the efficient production of pasture-raised ruminants in Brazil and worldwide. This study describes about fungi *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4) and their associated use in the control of gastrointestinal nematodes (GIN) from cattle. The *in vitro* studies involved mycelial growth rate indices (MGRI) observations of each fungus in three culture media, six temperatures and seven pH variations; compatibility between these isolates; electron and optical microscopy of their structures and interactions with nematodes and their joint nematicidal activities and isolated activities on cattle nematodes infective larvae (L3). The *in vivo* study evaluated the effects of oral administration of pellets containing fungal combination in the biological control of gastrointestinal parasitic nematodes in cattle and on pastures. The compatibility between SF53 and VC4 isolates was confirmed by direct confrontation, antibiosis and volatile compounds tests. Furthermore, the *in vitro* nematicidal evaluation showed that the best efficacy was when the two isolates were together used, with a 98.90% reduction in L3 number. The species *P. chlamydosporia* showed the highest growth rate in water agar medium at 20°C, whereas *M. sinense* showed a numerically better growth at 30°C. Fungi did not grow at 35 or 40°C. Surprisingly, the mycelial growth of both isolates was inhibited when the temperature was 35°C for 6 days but started again when the temperature was reduced to 25°C. The temperature of 40°C impeded fungal development in the laboratory. The pH observation was important to show that the pH variations in the gastrointestinal tract of bovines will not impede fungi development, since offering oral formulations to the animals is the most practical way for dispersing fungi in the faecal pats. Electron and optical microscopy showed the fungal structures that benefit their use in the biological control of nematodes and interactions with infective larvae of helminths. Nematode larvae held by *P. chlamydosporia* mycelium confirm its ability to prey upon larvae stages, despite being classified in the "ovicidal" group. In field evaluation, the L3 recovery in pasture of the group treated with the fungal combination was 91.7% and 86.4% lower than in pasture of the control group, respectively for samples collected at 20 cm and 40 cm from the feces. The eggs per gram of

feces (EPG) values in the months of May 2019 and January 2020 were lower ($p \leq 0.05$) in the treated group. Therefore, the fungi *M. sinense* and *P. chlamydosporia* are promising in the control of bovine gastrointestinal nematodes.

Keywords: Biocontrol agents. Compatibility. Mycelial growth. Nematophagous fungi. Helminths. Parasites. Pasture. Predators. Ruminants. Worms.

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LISTA DE SIGLAS E ABREVIATURAS

AA	Ágar Água
ABCBIO	Associação Brasileira de Empresas de Controle Biológico
ABIEC	Associação Brasileira das Indústrias Exportadoras de Carne
AC001	*Duddngtonia flagrans*
BDA	Batata Dextrose Ágar
BOD	Biochemical Oxygen Demand
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CMA	Corn Meal Agar
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
Embrapa	Empresa Brasileira de Pesquisa Agropecuária
FAPEMIG	Fundação de Amparo à Pesquisa do Estado de Minas Gerais
FPN	Fungos predadores de nematoides
GIN	Gastrointestinal nematodes
GPY	Glucose peptone yeast
IBGE	Instituto Brasileiro de Geografia e Estatística
I31	*Arthrobotrys robusta*
IVCM	Índice de Velocidade de Crescimento Micelial
L3	Larvas infectantes
MAPA	Ministério da Agricultura Pecuária e Abastecimento
MGRI	Mycelial Growth Rate Index
PDA	Potato dextrose agar
SD	Standard desviation
SF53	*Monacrosporium sinense*
SINDAN	Sindicato Nacional da Indústria de Produtos para Saúde Animal
VC4	*Pochonia chlamydosporia*
WA	Water Agar

LISTA DE SÍMBOLOS

®	Marca Registrada
≥	Maior igual
≤	Menor igual
μ	Micro
%	Porcentagem
Σ	Somatório

SUMÁRIO

1. INTRODUÇÃO

A criação de bovinos é uma atividade fundamental para a economia do Brasil. O país possui o maior rebanho comercial de gado do mundo (IBGE 2020), com grande potencial para produção de leite e carne. Um total de 43,3 milhões de bovinos foi abatido em 2019 e o volume de carne produzida foi equivalente a 10,49 milhões de toneladas de carcaça (ABIEC 2020). A produção da pecuária leiteira correspondeu a 25,01 bilhões de litros de leite (IBGE 2020).

Além disso, a criação dos animais predominantemente em pastagem (Beretta *et al*. 2002; Dias-Filho, 2016) favorece a ocorrência constante de infecções de nematoides gastrintestinais. No entanto, os produtores brasileiros não reconhecem a verminose como um problema parasitário dentro do sistema de produção (Delgado *et al*. 2009). Em contrapartida, a nematodiose gastrointestinal prejudica o desempenho dos animais e provoca perdas econômicas que já custaram cerca de US $ 7,11 bilhões/ano (Grisi *et al*. 2014).

Os gêneros de nematoides gastrintestinais mais relatados nos rebanhos brasileiros são: *Haemonchus*, *Cooperia*, *Oesophagostomum* (Oliveira *et al*. 2018; de Oliveira *et al*. 2021; Vieira *et al.* 2020a)*, Trichostrongylus e Ostertagia (*Ramos *et al. 2016).* O ciclo destes parasitas acontece em duas fases: uma fase de vida livre ocorrendo no ambiente que corresponde às fases de ovo até evoluir a larva infectante (L3) e uma fase de vida parasitária que desenvolve no animal e corresponde aos estágios de L3 até vermes adultos. A fase de vida livre inicia com a eliminação dos ovos nas fezes (Taylor *et al.* 2016). Estima-se que uma fêmea de *Haemonchus* possa produzir ate 10000 ovos/dia; *Cooperia* e *Oesophagostomum* cerca de 3000 ovos/dia; *Trichostrongylus* e *Ostertagia* cerca de 200 ovos/dia (Ueno e Gonçalves, 1998). No bolo fecal os ovos tornam-se embrionados e a larva passa de estágios iniciais até L3. A L3 migra do bolo fecal para a pastagem para ser ingerida pelo hospedeiro e, no trato gastrintestinal dos animais, inicia a fase parasitária até evoluir a vermes adultos (Girão *et al*. 1999).

O controle das verminoses tem sido feito predominantemente através do uso de anti-helmínticos (Kaplan, 2020). Delgado *et al*. (2009) constataram as avermectinas como o grupo químico mais utilizado nas fazendas brasileiras, seguida pelos imidotiazóis e benzimidazólicos. Não há restrições ao acesso aos anti-helmínticos comercialmente disponíveis, assim, o uso inadequado desses medicamentos não é raro (Delgado *et al*. 2009; Ramos *et al*. 2016). Tanto que, o maior faturamento da indústria de medicina veterinária brasileira se deve ao comércio de produtos antiparasitários (SINDAN, 2017), altamente

demandados na criação dos ruminantes. Porém, o uso dos compostos químicos para o controle elimina apenas a fase adulta dos parasitas alojados nos órgãos internos dos animais. Os estágios de vida livre, por outro lado, permanecem viáveis por semanas ou até meses no bolo fecal (Wang *et al*. 2020) e nas pastagens, dando continuidade ao ciclo de vida dos parasitas quando são novamente ingeridos pelos animais (Voinot *et al*. 2020). Além do mais, como os animais são frequentemente tratados excessivamente, há a aceleração da seleção de parasitas resistentes (Neves *et al*. 2014; Jaeger e Carvalho-Costa, 2017) e problemas significativos para a indústria pecuária (Ramos *et al*. 2016).

Como a administração de anti-helmínticos aos animais é insuficiente para o controle da reinfecção pelos helmintos, os estudos utilizando fungos nematófagos para o controle de formas infecciosas de nematoides gastrintestinais demonstraram que o uso de fungos é um manejo benéfico para o controle dos nematoides no ambiente (Araújo *et al*. 2021; Assis *et al*. 2012; Oliveira *et al*. 2018; Vieira *et al*. 2019). Os fungos nematófagos são predadores naturais de nematoides e são encontrados no solo em todo o mundo (Araújo *et al*. 2021; Braga e Araújo, 2014; Zhang *et al*. 2014) e por isso, a sua utilização nos sistemas de criação animal pode seguramente reduzir a quantidade de larvas infectantes no meio ambiente e favorecer a longevidade da ação das bases químicas (Araújo *et al*. 2021; Bilotto *et al*. 2018; Oliveira *et al*. 2018; Ortiz Pérez *et al*. 2017; Voinot *et al*. 2020).

O fungo *Pochonia chlamydosporia* é um parasito facultativo de ovos de nematoides e pertence ao grupo dos fungos helmintófagos "ovicidas". Possui como características morfológicas as hifas septadas que constituem seu micélio e produzem esporos em um saco fechado, denominado ascósporo. Possui vários formatos de conídios, variando de elíptico, globoso e algumas vezes bacilar, possui conidióforo pequeno e hifas diferenciadas, que em algumas situações podem ser eretas, clamidósporos com paredes espessas e morfologia tridimensional (Gams e Zaire 2001). As hifas desse fungo penetram a casca do ovo por meio de pequenos poros existentes na camada vitelínica. Isso causa alteração na permeabilidade da casca e leva à expansão do seu volume. Esse processo leva a uma divisão da camada vitelínica, e o fungo coloniza o conteúdo do ovo (Araújo *et al*. 2004). Há relatos na literatura que *P. chlamydosporia*, além da ação ovicida, parasita fêmeas de helmintos, as quais são colonizadas e digeridas completamente (Nordbring-Hertz *et al*. 2002; Lopes *et al*. 2007; Podestá *et al*. 2009).

O fungo *Monacrosporium sinense* preda nematoides através de redes adesivas e produz conídios medindo 25 - 30,5 µm de comprimento e 15 - 18 µm de largura, com um a três septos e clamidósporos esféricos a elipsoidais com 20 - 24 µm de diâmetro. Scholler *et al*.

(1999) baseados no tipo de armadilha produzida pelo fungo, propuseram a transferência da espécie para o gênero *Arthrobotrys*.

Por possuírem mecanismos de ação distintos sobre os nematoides estes fungos, quando utilizados conjuntamente, podem apresentar ação complementar e sinérgica no controle biológico de helmintos (Vieira *et al.* 2019). Entretanto, o crescimento em conjunto dos fungos pode ser inviável, assim, torna-se necessário conhecer a interação entre eles através de estudos de compatibilidade (Ayupe *et al*. 2016). Além de ser necessário compreender a influência das variáveis ambientais, como temperatura e pH, no crescimento e na manutenção desses fungos no ambiente.

2. REVISÃO DE LITERATURA

Aspectos biológicos dos fungos nematófagos e as formas de utilização

Os fungos nematófagos, muito estudados por serem inimigos naturais de nematoides, são microrganismos cosmopolitas, de ocorrência natural no solo, onde atuam como agente saprófita ou como agente parasita (Araújo *et al*. 2021; Zhang *et al*. 2014). A interação entre fungos nematófagos e nematoides induz a morfogênese e expressão gênica de virulência nesses fungos, sinalizando a transição do estágio saprofítico para o estágio fagocítico (Nordbring-Hertz B. 1988; Zhang *et al*. 2020). Com base em seu mecanismo de ação os fungos são classificados como fungos endoparasitas, oportunistas, produtores de toxinas e predadores (Braga e Araújo 2014; Zhang *et al*. 2014).

Fungos endoparasitas não produzem rede micelial extensa, são altamente dependentes do hospedeiro e possuem baixa capacidade saprofítica, o que dificulta o uso e produção *in vitro* (Braga e Araújo 2014). Estes fungos atacam os nematoides-alvos através de seus esporos, os quais, ou se aderem ao nematoide ou são engolidos por eles (Zhang *et al*. 2014).

Fungos oportunistas são capazes de colonizar ovos e fêmeas de nematoides, por meio da utilização de estruturas denominadas apressórios, e penetrar na forma infectante do nematoide (Dallemole-Giaretta *et al*. 2013). Enzimas hidrolíticas extracelulares, como quitinases e proteases, desempenham um papel importante durante a penetração da casca do ovo e levam à desintegração das suas camadas (Braga e Araújo 2014). Neste grupo de fungos os gêneros mais estudados são *Paecilomyces* e *Pochonia* (Zhang *et al*. 2014).

Fungos produtores de toxinas secretam toxinas que imobilizam o nematoide antes de iniciar o processo infeccioso. A maioria desses fungos são Basidiomycota embora os gêneros muito estudados *Paecilomyces* e *Pochonia* também produzam compostos nematicidas (Zhang *et al*. 2014).

Fungos predadores desenvolvem estruturas especializadas ao longo de suas hifas. As estruturas, com a função de capturar os nematoides, podem ser em forma de redes adesivas, botões adesivos, anéis constritores e não constritores (Nordbring-Hertz *et al*. 2006). A formação dessas armadilhas ocorre em resposta à presença do nematoide ou suas excretas, de compostos biológicos ou ainda, pode ser induzida por condições de estresse fisiológico (Nordbring-Hertz B. 1988; Zhang *et al*. 2014; Zhang *et al*. 2020). Além da formação dessas estruturas especializadas, os fungos predadores produzem clamidósporos intercalados nas hifas (Gronvold *et al*. 1996). Os clamidósporos são estruturas de resistência e garantem ao

fungo maior rusticidade (Herrera e Ulloa, 1990), favorecendo sua sobrevivência até que as condições ambientais sejam ideais para o seu desenvolvimento. É o grupo dos fungos mais pesquisado no controle biológico de nematoides e apresenta o maior potencial para industrialização (Braga e Araújo, 2014).

Como uma característica compartilhada por todos os tipos de fungos nematófagos, o reconhecimento dos hospedeiros e a adesão à cutícula dos nematoides ou cascas dos ovos pelos fungos são os primeiros passos na infecção. Para sobreviver e se reproduzir, os nematoides e os fungos predadores de nematoides (FPN) precisam lidar com sucesso com muitos estressores e demandas concorrentes no solo. A competição pode ser entre diferentes predadores de fungos, entre diferentes nematoides e entre FPN e nematoides (Zhang *et al*. 2020). Pesquisas descobriram que várias espécies de FPN frequentemente coexistem no mesmo nicho, sugerindo que elas provavelmente competem pela mesma presa em seus ambientes naturais. Alguns nematoides são capturados/colonizados por mais de um FPN ao mesmo tempo (Yang *et al*. 2020). Houve também estudos em que foram encontradas evidências de competição entre FPN (Dijksterhuis *et al*. 1994).

Os fungos que se mostraram mais promissores no biocontrole são dos gêneros *Arthrobotrys, Duddingtonia, Monacrosporium* e *Pochonia* (Braga *et al*. 2014). A forma mais comum e prática de utilização dos fungos nematófagos em condições a campo é através da alimentação dos animais (Araújo *et al*. 1999). Como os fungos atuam na fase de vida livre dos parasitos uma maneira para que eles fossem dispersos no bolo fecal, onde se encontram os ovos e larvas dos nematoides, seria pela sua ingestão (Araújo *et al*. 2004b). Entretanto, é necessário que o fungo resista ao estresse da passagem pelo trato gastrintestinal dos animais e chegar viável às fezes para predar nematoides (Larsen *et al*. 1992). Por isso, testar a capacidade da resistência dos fungos à passagem pelo trato gastrintestinal dos animais é o primeiro passo nos estudos *in vivo* para avaliar a viabilidade da utilização de um fungo nematófago no controle de parasitos de uma determinada espécie animal (Araújo *et al*. 1996; Llerandi-Juárez e Mendoza-de-Gives, 1998; Araújo *et al*. 1999; Oliveira *et al*. 2018; Vieira *et al*. 2020b; Rodrigues *et al*. 2021). Além disso, o crescimento rápido e produção abundante de micélio são também dois importantes fatores para a disseminação e sobrevivência dos fungos em condições ambientais, embora o crescimento micelial não esteja relacionado com a capacidade de um isolado em predar nematoides (Dackman *et al*. 1987).

A administração dos fungos aos animais pode ser realizada na forma de clamidósporos, conídios ou massa micelial. No Brasil, o desenvolvimento de formulações fúngicas por meio de matriz de alginato de sódio contendo micélio fúngico de diversos

isolados tem se mostrado uma boa alternativa de veiculação e administração de fungos (Araújo *et al*. 2004b; Oliveira *et al*. 2018; Vieira *et al*. 2020a) com bons resultados em condições laboratoriais e a campo, porém sua utilização para fins comerciais não é viável devido ao alto custo para produzi-los.

O uso de suspensão oral também abrange diversos trabalhos. Araújo *et al*. (1998) demonstraram que a administração oral de conídios do fungo nematófago *Arthrobotrys robusta*, duas vezes por semana, durante quatro meses foi capaz de reduzir o valor do OPG (ovos por grama de fezes) e a quantidade de vermes em bovinos infectados naturalmente com nematoides gastrintestinais. Uma suspensão aquosa contendo clamidósporos de um isolado mexicano de *Duddingtonia flagrans* foi avaliado por Mendoza de Gives *et al*. (1998) no controle de larvas infectantes de *Haemonchus contortus*. Uma dose única contendo 11.350.000 clamidósporos administradas via oral em ovelhas resultou em uma redução de até 88% da população do nematoide nas fezes.

O fornecimento de blocos energéticos (a base de melaço, ureia, levedura, óleo, fósforo, cálcio e outros minerais) contendo clamidósporos de *Duddingtonia flagrans* foi avaliado por Sagüés *et al*. (2011) os quais obtiveram sucesso no controle de nematoides gastrintestinais de ovinos. Já Aguilar-Marcelino *et al*. (2016) formularam péletes nutricionais para cordeiros com 7% de farelo de sorgo, 51% de farelo de soja, 20% de farelo de trigo, 18% de melaço, 2% de mistura mineral comercial, 2% de cálcio e a inclusão de clamidósporos de *Duddingtonia flagrans* e observaram redução de 70% de larvas nas fezes.

Arroyo *et al*. (2016) avaliaram a capacidade dos clamidósporos do fungo ovicida *Mucor circinelloides* de resistir ao processo de peletização industrial. Os esporos fúngicos foram adicionados no processo de mistura dos componentes da ração, os quais foram, posteriormente, submetidos à temperatura de 72ºC. Os clamidósporos do *M. circinelloides* foram capazes de resistir ao desafio térmico do processo de peletização, mantendo sua capacidade de infecção e destruição de ovos de nematoides. Hernández *et al*. (2016) utilizaram péletes contendo clamidósporos dos fungos *Mucor circinelloides* e *Duddingtonia flagrans* (ambos incorporados ao mesmo pélete) na alimentação de cavalos. Os fungos foram submetidos ao estresse térmico inerente ao processo de peletização e foram capazes de resistir à temperatura de 75ºC por 90 segundos sem perder suas atividades ovicida e predadora, respectivamente.

A utilização de grãos contendo fungos nematófagos previamente crescidos e fornecidos na alimentação animal, para avaliar redução de formas infectantes de parasitos nas fezes também é eficiente (Buzati *et al*. 2015) e demonstra-se como uma forma prática

comercialmente (Braga *et al*. 2020). O fungo *Duddingtonia flagrans* (BioVerm ®) crescido em arroz é o primeiro produto biológico brasileiro aliado ao controle de formas infectantes de nematoides gastrintestinais de animais criados em pastagens (Braga *et al*. 2020). Estudo conduzido com o produto mostrou que o uso da formulação Bioverm® foi eficaz na descontaminação das pastagens e consequentemente na ocorrência de reinfecção por nematoides. Além do mais, os animais tratados com Bioverm® apresentaram menor carga parasitária e maior ganho de peso (de Oliveira *et al*. 2021; Holsback *et al*. 2021).

No entanto, para que o uso dos fungos ofereça uma solução eficaz no controle de nematoides, a dinâmica do ecossistema, onde esses agentes são introduzidos, precisa ser continuamente avaliada. Os fatores ambientais são muito importantes na determinação da eficácia dos fungos nematófagos, pois interferem diretamente na formação do micélio (Gronvold *et al*. 1999). Vieira *et al*. (2020b) observaram que os fungos nematófagos *Duddingtonia flagrans, Arthrobotrys cladodes* e *Pochonia chlamydosporia* não foram limitados por variações de temperatura (15, 20, 25, 30 e 35°C) para o controle biológico de nematoides parasitas de bovinos em condições *in vitro*. No entanto, segundo os mesmos autores, estes fungos apresentaram maior crescimento, produção de clamidósporos e atividade nematicida nas temperaturas intermediárias (20, 25 e 30°C) do que nas extremas de 15 e 35°C. Bilotto *et al*. (2018) avaliaram a ação predatória de *Duddingtonia flagrans* sobre nematoides gastrintestinais em bezerros em diferentes épocas do ano. No verão houve uma redução de 60-90% de larvas tanto nas áreas sombreadas quanto nas áreas ensolaradas. Porém, no inverno não houve redução significativa dos nematoides nas duas condições. Isso pode ser resultado de um maior índice de precipitação e menor eficiência em baixas temperaturas para a germinação de clamidósporos, desenvolvimento de estruturas fúngicas, crescimento micelial e capacidade saprofítica competitiva (Bilotto *et al*. 2018).

Em todo o mundo, os helmintos gastrintestinais são um dos maiores problemas enfrentados pela criação de animais em sistema a pasto. Nos últimos 30 anos, nossa compreensão sobre controle biológico aplicado aos nematoides evoluiu muito. Do ponto de vista ecológico, a interação entre os fungos nematófagos e os nematoides podem contribuir para a manutenção e estabilidade das formas infectantes dos helmintos gastrintestinais parasitos de animais presentes nas pastagens. Portanto, pesquisas futuras na busca por novas formulações, associação de fungos de diferentes grupos, além da extração de moléculas de fungos nematófagos para o controle de helmintos são desejáveis (Araújo *et al*. 2021).

3. JUSTIFICATIVA E OBJETIVOS

Os animais criados em sistema de pastagem estão continuamente expostos a formas infectantes (L3) de helmintos devido à falta de um manejo ambiental eficaz, e os fungos nematófagos podem ser um complemento importante para o controle de nematoides gastrintestinais. O sucesso para o emprego desses fungos depende do seu crescimento, colonização, sobrevivência no ambiente onde serão instalados e eficiência na redução de L3. Portanto, fatores como composição do meio, temperatura, pH e compatibilidade podem influenciar diretamente a atuação desses agentes de biocontrole.

3.1 Objetivo geral

Avaliar a compatibilidade de crescimento entre os fungos nematófagos *M. sinense* e *P. chlamydosporia* em condições laboratoriais e a ação conjunta desses fungos na redução dos helmintos gastrintestinais de bovinos.

3.2 Objetivos específicos

- Verificar a compatibilidade entre os fungos *M. sinense* e *P. chlamydosporia* com os testes de confrontação direta, antibiose e de compostos voláteis em laboratório;
- Avaliar o crescimento dos fungos *M. sinense* e *P. chlamydosporia* em diferentes meios de cultura, condições de temperatura e pH;
- Observar as estruturas fúngicas e as interações com larvas infectantes de nematoides gastrintestinais de bovinos através de microscopia óptica e eletrônica de varredura;
- Quantificar a carga parasitária dos animais entre o grupo tratado e controle e a infestação da pastagem após a administração da associação fúngica em formulação peletizada.

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5. CAPÍTULO 1

In vitro compatibility and nematicidal activity of *Monacrosporium sinense* and *Pochonia chlamydosporia* for biological control of bovine parasitic nematodes

In vitro compatibility and nematicidal activity of *Monacrosporium sinense* and *Pochonia chlamydosporia* for biological control of bovine parasitic nematodes

Abstract

The use of nematophagous fungi is an alternative for the biological control of nematodes in ruminants. In this study, the compatibility of joint growth of the fungi *Monacrosporium sinense* and *Pochonia chlamydosporia* and the joint nematicidal activity of these fungal isolates on bovine infective larvae were evaluated. For that, tests of direct confrontation, the effect of volatile compounds and antibiosis were conducted. In order to carry out the tests, the fungi were inoculated in potato dextrose agar culture medium and, after the incubation period, the growth of the colonies, the formation of an inhibition halo and the effect of volatile metabolites were verified. The compatibility between fungi isolates *M. sinense* and *P. chlamydosporia* was confirmed and the nematicidal evaluation proved the best effectiveness was when both were used together, with a 98.90% reduction in the number of bovine nematode infective larvae under *in vitro* conditions. It was concluded that *M. sinense* and *P. chlamydosporia* presented synergistic action, suggesting that the joint application of the fungi increases the effectiveness of biological control of bovine infective larvae.

Keywords: Antibioses, biocontrol, direct confrontation, helminths, interactions, volatile metabolites

Key Findings

- The fungi presented homogeneous growth in the direct confrontation tests.
- In the antibiosis tests there was no inhibition of halo formation among them
- There was no production of metabolites capable of inhibiting their growth together.
- The fungi association reduced in 98.90% the number of infective nematode larvae.

Introduction

The climate conditions and the territorial size are peculiarities that define an important characteristic of livestock in Brazil: a large part of the herd is raised on pastures, thus making possible one of the lowest cattle production costs in the world (Embrapa 2019). In addition, Brazil offers a product with the potential to conquer more demanding markets, the so-called ‘green ox’ or ‘grass-fed beef’ (Dias-Filho 2016). However, pasture-reared ruminants populations are exposed to several gastrointestinal nematodes which are a cause of significant losses in animal production worldwide (Charlier *et al*. 2020; Szewc *et al*. 2021). At the same

time, difficulties encountered in the chemical control of nematodosis (Avramento *et al*. 2020), have encouraged researchers to develop new ecologically appropriate and sustainable technologies in pasture production systems (Szewc *et al*. 2021; Velde *et al*. 2018). Among them, the use of nematophagous fungi in the biological control of gastrointestinal nematodes in ruminants has been explored in several studies around the world, as it contributes directly to reduce the use of chemotherapy in cattle production systems (Braga and Araújo 2014). In animal production, biotechnology can be used to increase food production, product quality and system sustainability (Baker *et al*. 2020; Carnevali *et al*. 2019; Yuan *et al*. 2019). The product Bioverm®, with a commercialisation licence granted by the Ministério da Agricultura Pecuária e Abastecimento (MAPA 2019), contains structures of the fungus *Duddingtonia flagrans* and enters the Brazilian market as a new tool for nematodosis control programmes and thus contributes to improving the efficiency of animal production in a way that is non-toxic to the environment.

Nematophagous fungi are naturally present in the environment and comprise a variety species with the ability to prey on, or render unviable, nematodes by enzyme production and then to use the nematodes as a source of nutrients (Van Ooij 2011). The species *Monacrosporium sinense* predates nematode larvae through adhesive networks (Campos *et al*. 2007). The species *Pochonia chlamydosporia* is an optional parasite of eggs and females of nematodes (Dallemole-Giaretta *et al*. 2013) and belongs to the group of nematophagous fungi called ‘ovicides’. The joint use of different isolates of nematophagous fungi may have a complementary and synergistic action in the biological control of helminths (Vieira *et al*. 2019). For this, the introduction of a fungus with excellent interaction capacity and adaptation to the microenvironment is relevant to the success of a parasite control programme.

This research aimed to evaluate the compatibility of joint growth of the fungi *M. sinense* and *P. chlamydosporia* and the joint nematicidal activity of these fungal isolates on infective larvae of bovine nematodes.

Materials and methods

The fungal isolates *M. sinense* (SF53) and *P. chlamydosporia* (VC4) are part of the collection of the Parasitology Laboratory in the Veterinary Department of the Federal University of Viçosa, Brazil, where they are preserved on rice grains in silica gel tubes at 4°C in the dark. The rice grains containing structures of the fungus isolates were placed to grow separately in plates containing 2% potato dextrose agar medium (2% PDA).

Antagonism test in direct confrontation

Discs of potato dextrose agar medium of 5 mm diameter and containing *P. chlamydosporia* mycelia were placed at a distance of 1 cm from the edge of Petri dishes (9 cm in diameter) containing 2% potato dextrose agar medium (2% PDA). The plates were stored in the dark for 10 days at 26 °C in an incubator chamber with biochemical oxygen demand (BOD). After this period, mycelium discs of *M. sinense* were placed on the agar opposite the colony of *P. chlamydosporia*. For the control group, colonies of the same fungus were compared. The confronted colonies were incubated in the dark for eight days at 26°C in an incubator chamber (BOD). For the evaluations, the adapted scale of notes, proposed by Bell et al. (1982) was used: 1 – complete colonisation of the plaque by *P. chlamydosporia*; 2 – colonisation of 2/3 of the plaque by *P. chlamydosporia*; 3 – colonisation of 50% of the plaque per fungus; 4 – colonisation of 2/3 of the plaque by *M. sinense*; 5 – complete colonisation of the plaque by *M. sinense*. Ten repetitions were performed per treatment.

Antibiosis test

Dialysis membrane discs (SIGMA®) were placed on the surface of the 2% PDA culture medium in Petri dishes of 9 cm diameter (Figure 1A). Subsequently, 5 mm diameter agar discs of the mycelia of *P. chlamydosporia* (VC4) and *M. sinense* (SF53) were placed separately in the centre of distinct plates (Figure 1B). The colonies were incubated in the dark for 96 hours at 26°C in an incubator chamber (BOD). The growth area of the colonies was demarcated externally at the bottom of the plates and the dialysis membrane together with the respective colony was removed (Figure 1C). Then, the plates were inverted and 1 mL of chloroform was added to the bottom in order to eliminate possible structures of the fungus (Figure 1D). After the product had evaporated, the plates were left for 30 min under direct irradiation of ultraviolet light in a laminar flow chamber (Figure 1E). Then, an aqueous suspension containing mycelia of *P. chlamydosporia* was added to the surface of the culture medium in the plates where the fungus *M. sinense* grew and a suspension of *M. sinense* where *P. chlamydosporia* grew (Figure 1F). These suspensions were obtained from colonies previously grown in 2% PDA culture medium for 10 days in the dark at 26 °C in an incubator chamber (BOD).

The control group consisted of cultivation of *P. chlamydosporia* and a subsequent suspension culture containing mycelia of *P. chlamydosporia*, the same procedure being carried out for the control group of *M. sinense*. The plates were kept at 26 °C in an incubator

chamber (BOD) for 10 days in the dark and after this period it was observed whether there was a growth inhibition halo of *P. chlamydosporia* formed by *M. sinense*, and vice versa. In order to ensure that the method generated reliable information about the samples, 10 repetitions were performed per treatment.

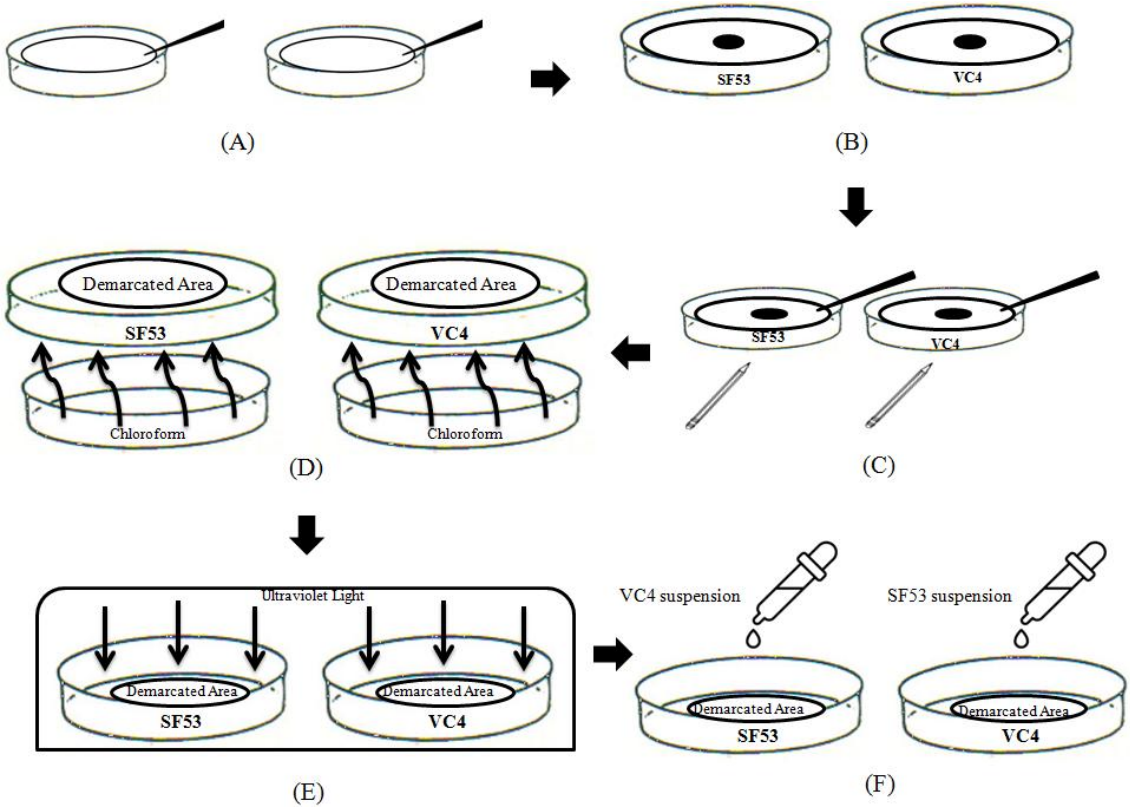

Figure 1: Antibiosis test represented by diagram.

Effect of volatile compounds

Petri dish covers of 9 cm in diameter containing 2% PDA culture medium, were positioned one above the other according to the technique described by Bharat *et al*. (1980). A 5 mm diameter disc containing *M. sinense* mycelia was added to the lower plate and a 5 mm diameter disc containing *P. chlamydosporia* mycelia was added to the top plate. In another treatment, a 5 mm diameter disc containing mycelia of *P. chlamydosporia* was added to the lower plate and a 5 mm diameter disc containing *M. sinense* mycelia was added to the top plate. For the control group, *P. chlamydosporia* was cultivated in the lower and top plate; the same procedure was carried out for *M. sinense* (Figure 2A). The plates were laterally sealed with a plastic membrane and kept at 26°C in an incubator chamber (BOD) for 15 days and in the dark (Figure 2B). For the evaluations, the colony area was measured and compared with

the control (Figure 2C). Ten repetitions were performed per treatment and Student's ‘t’ test was used to compare the areas of the colonies at a significance level of 5%.

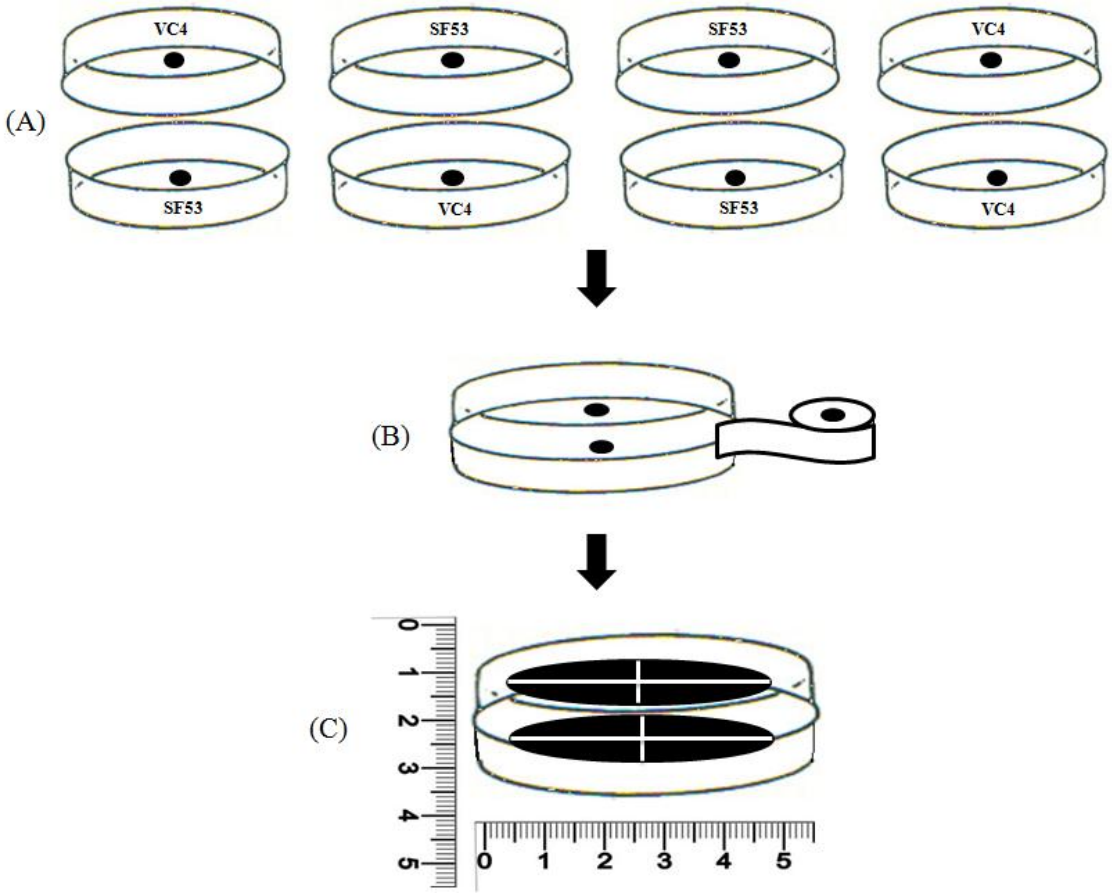

Figure 2: Effect of volatile compounds test represented by diagram.

Production of pellets in sodium alginate containing mycelia of the fungi *M. sinense* and *P. chlamydosporia*

For the induction of fungal mycelium formation, agar fragments of approximately 5 mm in diameter containing mycelia and fungal spores of the isolates VC4 and SF53 were transferred separately to Erlenmeyer flasks containing 150 mL liquid medium of glucose, peptone and yeast extract (GPY). The fungi were grown at pH 6.5, 120 rpm agitation for 24 h in the dark at 26 °C for 21 days. After this period, the mycelia were harvested with a platinum loop and weighed on an analytical scale for the manufacture of pellets. The pellets were produced in a matrix of sodium alginate, according to the technique described by Walker and Connick (1983) and modified by Lackey *et al*. (1993).

In vitro evaluation of nematicidal activity of fungi towards infective larvae of gastrointestinal nematodes

Forty Petri dishes of 9 cm diameter containing 2% water–agar (2% WA) were used, divided into four groups. In the first group, 0.2 g of pellets containing *P. chlamydosporia* were added to the plates; in the second group, 0.2 g of pellets containing *M. sinense* (SF53) were added to the plates; in the third group, 0.2 g of pellets containing the association of *M. sinense* and *P chlamydosporia* were added to the plates; and in the fourth group, 0.2 g of pellets without fungus (control) were added to Petri dishes containing 2% WA. Into each plate were poured 1000 infective larvae (L3) of bovine nematodes, corresponding to the genera *Haemonchus* sp., *Cooperia* sp. and *Oesophagostomum* sp. in the proportions ($\pm$SD) of 50% ($\pm$11), 44% ($\pm$5) and 6% ($\pm$5) respectively. The plates were kept in an incubator chamber at 26 °C in the dark for a period of 7 days. On the 7th day, the L3 were recovered by emptying the plate contents of into a Baermann funnel, with water at 42–45 °C and waiting for 12 hours before decantation. Recovered infectious larvae were counted and identified according to Keith's criteria (1953) under a light microscope (100X magnification), obtaining the average of the non-predated larvae by genus in the control group, and the average of non-predated larvae by genus in the treated group. The percentage reduction of larvae in the treated group in relation to the control was calculated according to the following formulae:

$$\text{Reduction (\%)} = \frac{\text{(mean of control larvae – mean of treatment larvae)}}{\text{mean of control larvae}} \times 100$$

The recovered L3 averages, the L3 percentages of each genus and the reduction percentages were transformed into log (x + 1) and compared by Tukey's test at a significance level of 5% using IBM SPSS 2.0 software.

Results

The growth of *M. sinense* and *P. chlamydosporia* in association and separately are shown in Figure 3. According to the Bell scale adapted for this study, the isolates confronted in the in vitro antagonism test were classified as grade '3', in which the growth of each fungus occupied 50% of the plate.

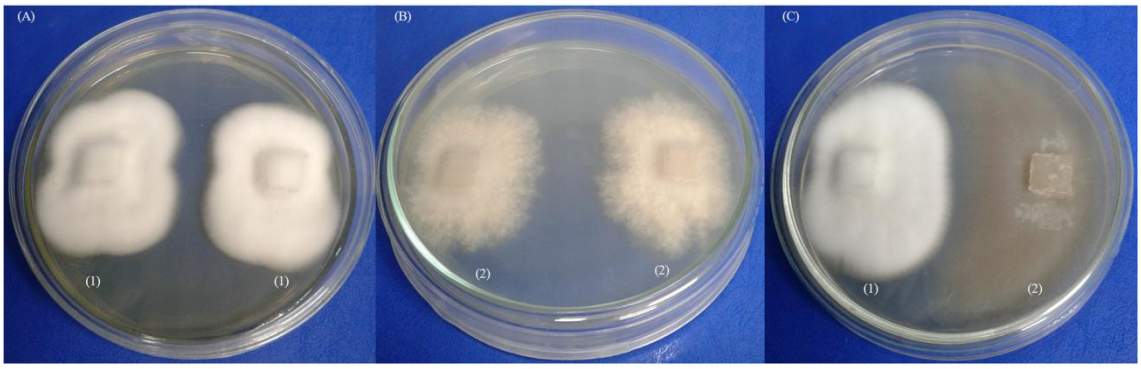

Figure 3: Direct confrontation test: (A) *Pochonia chlamydosporia* (1) and *Pochonia chlamydosporia* (1); (B) *Monacrosporium sinense* (2) and *Monacrosporium sinense* (2); (C) *Pochonia chlamydosporia* (1) and *Monacrosporium sinense* (2).

The non-formation of an inhibition halo between the fungi *M. sinense* and *P. chlamydosporia* observed in the antibiosis test demonstrated that the isolates did not produce substances that would interfere with mycelial growth.

Table 1 shows the average values of the mycelial growth area in the upper parts of the plates of the fungi *M. sinense* and *P. chlamydosporia* submitted to the volatile metabolites effect test, in which no differences were observed in the mycelial growth area of any of the isolates tested together ($p \geq 0.05$).

Table 1. The average values and standard deviation of the mycelial growth area, in the upper parts of the Petri dishes, 9 cm in diameter, containing 2% potato-dextrose-agar medium (2% PDA), of the fungi *Pochonia chlamydosporia* (VC4 isolate) and *Monacrosporium sinense* (SF53 isolate) submitted to the volatile metabolite effect test.

Monacrosporium sinense		***Pochonia chlamydosporia***	
Treatments	**Area(cm^2)**	**Treatments**	**Area(cm^2)**
SF53 *versus* VC4	$49{,}99^a \pm 0.53$	VC4 *versus* SF53	$14.76^b \pm 1.58$
SF53 *versus* SF53	$50.12^a \pm 0.39$	VC4 *versus* VC4	$12.94^b \pm 2.67$

Different letters in the same column indicate the difference between the data ($P \leqslant 0.05$). SF53: *Monacrosporium sinense*; VC4: *Pochonia chlamydosporia.*

The nematicidal activities of the fungi were demonstrated by means of infective larvae (L3) of parasitic bovine nematodes recovered after 7 days. The percentage L3 reduction in the group containing the association of *M. sinense* and *P. chlamydosporia* (98.90%) was higher than the percentage of the group containing *M. sinense* alone (82.20%), which was higher than the percentage of the group containing *P. chlamydosporia* alone (34.70%), as shown in Figure 4.

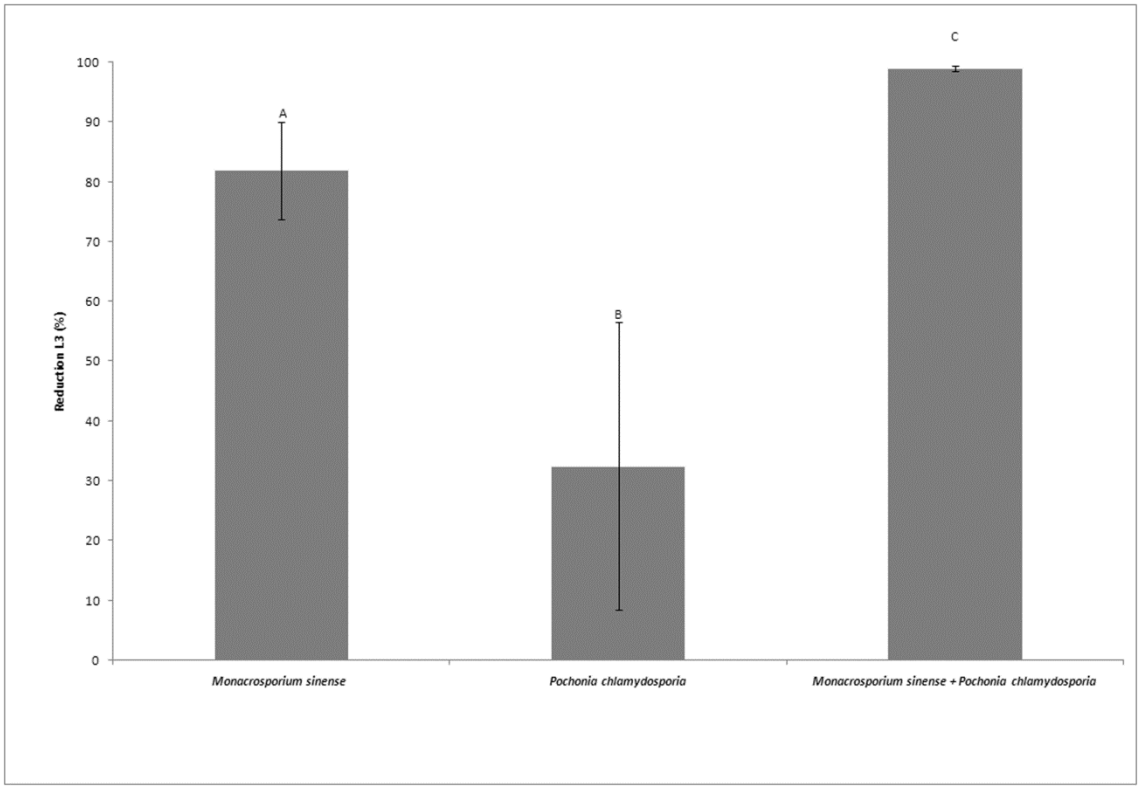

Figure 4: The percentage reduction of infectious larvae (L3) of gastrointestinal parasitic nematodes of cattle recovered from plates with 2% water-agar medium (2% WA), after 7 days of interaction, of the fungi *Monacrosporium sinense* (SF53 isolate), *Pochonia chlamydosporia* (VC4 isolate) and the association of the fungi *Monacrosporium sinense* and *Pochonia chlamydosporia* (SF53+VC4). Different letters indicate the difference between the values ($p \leq 0.05$).

Table 2 shows the average values of infectious larvae (L3) of cattle recovered from the plates with 2% WA medium, containing the association of the fungi *M. sinense* and *P. chlamydosporia* (SF53 + VC4), *P. chlamydosporia* (VC4) alone and *M. sinense* (SF53) alone, as well as the percentage distribution of recovered L3 genera. Compared to the control group without fungus, the three groups that contained nematophagous fungi showed lower values of recovered L3 ($p \leq 0.05$). The group that contained the association *M. sinense* and *P. chlamydosporia* showed the lowest recovery of L3 of all three groups. The group that contained *M. sinense* alone showed lower recovery of L3 compared to the group that contained *P. chlamydosporia* alone ($p \leq 0.05$).

Table 2. The mean values and standard deviations of the number of infective larvae (L3) recovered from plates with 2% water-agar medium (2% WA), after 7 days of interaction, containing the association of nematophagous fungi *Monacrosporium sinense* and *Pochonia chlamydosporia* (SF53+VC4), *P. chlamydosporia* (VC4) and *M. sinense* (SF53), as well as the percentage distribution of recovered L3 genera.

Group	L3 Recovered	*Haemonchus* %	*Cooperia* %	*Oesophagostomum* %
Control	$770,3^{A}(\pm 142,30)$	$51,23^{A}$ ($\pm$4,52)	$42,95^{A}$ ($\pm$4,07)	$5,82^{AB}$ ($\pm$1,82)
SF53	$137,1^{B}(\pm 62,70)$	$43,21^{A}$ ($\pm$7,07)	$54,09^{B}$ ($\pm$6,99)	$2,70^{AC}$ ($\pm$2,11)
VC4	$484,1^{C}$ ($\pm$133,50)	$49,80^{A}$ ($\pm$6,09)	$41,00^{A}$ ($\pm$7,62)	$9,19^{B}$ ($\pm$2,59)
SF53+VC4	$8,1^{D}$ ($\pm$3,4)	$50,96^{A}$ ($\pm$15,45)	$47,61^{AB}$ ($\pm$16,70)	$1,43^{C}$ ($\pm$4,52)

Different letters in the same column indicate the difference between the data ($p \leq 0,05$). SF53: *Monacrosporium sinense*; VC4: *Pochonia chlamydosporia.*

There was no significant variation ($p \geq 0.05$) in the percentages of L3 of the *Haemonchus* genus between the four groups. The percentage of L3 of the genus *Cooperia* was higher in the group containing only *M. sinense* than in the control and VC4 groups. The percentage of L3 of the genus *Oesophagostomum* recovered from the group containing the fungal association was lower compared to the control group, while the recovery of *Oesophagostomum* in the *P. chlamydosporia* group was higher than in the group containing only *M. sinense*.

Discussion

To multiply, microorganisms depend on the availability of nutrients, space and oxygen. In addition, they need to adapt to the existing competition with any microbiota. Thus, interactions between fungi can be positive when synergistic multiplication occurs, or negative when inhibition of one of the agents occurs. Thus, in this work, the viability of the association

between *M. sinense* and *P. chlamydosporia* was evaluated in order to use this combination of fungi in the biological control of gastrointestinal nematodes in animals.

The present study showed that *M. sinense* and *P. chlamydosporia* in direct confrontation showed homogeneous growth without overlapping colonies. This result suggests that these isolates can present very promising results in field tests. In addition, no significant volatile compound production was found by any of the fungi, since no degree of antagonism was seen in the in vitro test for volatile metabolites. There was no inhibition halo between the fungi *M. sinense* and *P. chlamydosporia* in the antibiosis test, demonstrating that the isolates did not produce substances that would interfere with each other's mycelial growth. The performance of compatibility tests is a way of understanding the capacity for interaction between fungi and reinforces the possibility of the joint use of these isolates in further studies. The fungi *Arthrobotrys cladodes* and *P. chlamydosporia* evaluated by Vieira *et al*. (2019) also presented themselves as compatible species for joint growth, since, in direct confrontation, each fungus colonized 50% of the plate, which revealed the possibility of other isolates with joint survivability. On the other hand, in contrast to our results and to those previously described, Ayupe *et al*. (2016) reported that *Arthrobotrys robusta* (I31) competes with and antagonises *Duddingtonia flagrans* (AC001), which reinforces the need to perform compatibility tests for the successful use of fungal associations.

The species *M. sinense* and *P. chlamydosporia* are commonly found in the soil and although these isolates have already been tested for their ability to be included in nematode biological control strategies (Campos *et al*. 2007; Vieira *et al*. 2020a; Vieira *et al*. 2020b), the combined use of these fungi had not yet been studied. In addition, the success of their use in the biological control of parasites requires detailed knowledge of the agents and their interactions in the environment.

Regarding the suppression of the high-density nematode population, the association of *M. sinense* and *P. chlamydosporia* proved to be more efficient than the other treatments, reducing by 98.90% the number of infective larvae of bovine gastrointestinal parasites, while the reductions in groups containing *M. sinense* or *P. chlamydosporia* alone were 82.20% and 34.70%, respectively. The predator fungi *Monacrosporium sinense* modified its hyphae in adhesive networks holding the nematode (Campos *et al*. 2007). Diversily, *P. chlamydosporia* produced extracellular enzymes which were capable of causing cuticle hydrolysis and death of nematode larvae (Yang *et al*. 2013) and it produced toxins with nematicidal activity (Mukhtar and Pervaz 2003) which made it possible to achieve a satisfactory nematicidal

activity index (Vieira *et al*. 2020a). This reinforces the hypothesis that the association of fungi with different nematocidal activities has a greater chance of success in controlling parasites than the use of a single species. This was also reported in the compatibility between *P. chlamydosporia* and *A. cladodes* determined by Vieira *et al*. (2019), which resulted in a higher percentage reduction in the number of bovine infective larvae when compared their use in isolation. Efficiency is essential for the success of a biocontrol and its market acceptance.

When testing the effect of the fungal association on larvae of the genus *Haemonchus*, there was no significant difference between the four groups. The combination of fungi studied by Vieira *et al*. (2019), showed greater selectivity for the genus *Haemonchus*. The fact that there were variations in the percentage reduction of larvae of other genera can be justified by the predominance of the nematode genus, motility and cuticle composition. (Mendoza-de-Gives *et al*. 1999; Oliveira *et al*. 2018; Vieira *et al*. 2019).

This research made it possible to confirm the compatibility between the isolates *Monacrosporium sinense* and *Pochonia chlamydosporia*, which demonstrated their best efficiency when used together to control bovine infective larvae under *in vitro* conditions.

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Author Contribution

Isabela and Jackson Victor conceived and designed the study. Isabela conducted data gathering. Ítalo performed statistical analyses. Isabela, Ítalo, Artur and Jackson Victor wrote the article.

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Conflicts of Interest

The authors declare there are no conflicts of interest.

Ethical Standards

'Not applicable' here.

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6. CAPÍTULO 2

Evaluation of nematophagous fungal mycelial growth and interactions with bovine gastrointestinal parasitic nematodes

Evaluation of nematophagous fungal mycelial growth and interactions with bovine gastrointestinal parasitic nematodes

Abstract

Previous research has shown an increased action on helminth biological control by fungal combinations. This study characterised the temperature and pH conditions necessary for better mycelial growth of *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4). In addition, electron and optical microscopy showed the fungal structures that benefit their use in the biological control of nematodes and interactions with infective larvae of helminths. Nematode larvae held by *P. chlamydosporia* mycelium confirm its ability to prey upon larvae stages, despite being classified in the "ovicidal" group. The species *P. chlamydosporia* showed the highest growth rate in water agar medium at 20°C, whereas *M. sinense* showed a numerically better growth at 30°C. Fungi did not grow at 35 or 40°C. Surprisingly, the mycelial growth of both isolates was inhibited when the temperature was 35°C for 6 days but started again when the temperature was reduced to 25°C. The pH observation was important to show that the pH variations in the gastrointestinal tract of bovines will not be harmful to fungi, since offering oral formulations to the animals is the most practical way for dispersing fungi in the faecal pats. *In-vitro* studies facilitate the exploration of biological control agents. The use of nematophagous fungi is a viable solution in the control of gastrointestinal nematodes and needs to be further improved.

Key words: Biological control, environmental conditions, helminths, *Monacrosporium sinense*, *Pochonia chlamydosporia*, predation

Key Findings

- *P. chlamydosporia* preys upon nematode larvae
- *M. sinense* showed excellent growth at 30°C.
- *P. chlamydosporia* showed highest growth rate in water-agar medium at 20°C.
- A temperature of 40°C impeded fungal development in the laboratory.
- Mycelium growth was not affected by ambient pH.

Introduction

For long-term effective parasite control in animal production systems, the combination of management strategies is required. Nematophagous fungi can be an important adjunct to the traditional gastrointestinal nematode control (Araújo *et al*. 2021; Canhão-Dias *et al*. 2021; Holsback *et al*. 2021; Szewc *et al*. 2021).

Nematophagous fungi are natural nematode predators in soil (Braga and Araújo 2014; Zhang *et al*. 2014), where they develop parasitic or predatory relationships with nematodes and are classified as nematode-trapping, opportunistic or ovicidal, endoparasitic, toxin producers and producers of specific attack devices (Araújo *et al*. 2021). The fungus *Pochonia chlamydosporia* parasitises eggs and female nematodes (Dallemole-Giaretta et al. 2013; Vieira et al. 2019) and belongs to the nematophagous fungal group known as "ovicidal fungi" (Araújo *et al*. 2021), and the species *Monacrosporium sinense* predates nematode larvae through adhesive networks (Campos *et al*. 2007). Each fungus attacks nematodes at different stages, and a compatibility study between *M. sinense* and *P. chlamydosporia* has demonstrated greater success in nematode control by their combined use (Oliveira *et al*. 2021a). However, fungi need to find suitable environmental conditions, such as an optimum temperature, for efficient predatory activity of nematode infecting forms (Vieira *et al*. 2020). Thus, basic and applied studies about the biological variables influencing the development of these microorganisms are required (Li *et al*. 2019; Vieira *et al.* 2020; Ocampo-Gutiérrez *et al*. 2021).

Studies on nematophagous fungi controlling gastrointestinal nematode infective stages, particularly in pasturing ruminants (Mendoza-de-Gives *et al*. 2018; Oliveira *et al*. 2018; Vieira *et al*. 2019; Voinot *et al*. 2020), have provided substantial evidence that these microorganisms can efficiently be used in nematode control (ABCBIO 2016; Araújo *et al*. 2021; Braga *et al*. 2020; Oliveira *et al*. 2021b). In addition to their predatory activity, their

rapid growth is another important factor for their survival and spread in the environment (Anan'ko and Teplyakova, 2011; Li *et al*. 2019; Vieira *et al*. 2020) and is therefore of commercial interest (Braga et al. 2020; Oliveira *et al*. 2021b); however, related data are scarce. In this context, this study investigated the optimal pH and temperature conditions of *M. sinense* and *P. chlamydosporia* growth under laboratory conditions, with the aim to enhance the use of fungi in biological nematode control strategies.

Materials and methods

The fungi *Monacrosporium sinense* (SF53 isolate) and *Pochonia chlamydosporia* (VC4 isolate) used in this study are part of the collection of the Parasitology Laboratory of the Veterinary Department, Federal University of Viçosa, where they are kept at 4°C in the dark in test tubes containing 2% corn meal agar medium (2% CMA). These isolates have been obtained from Brazilian agricultural soil in the municipality of Viçosa (Zona da Mata, Minas Gerais), collected using the soil-sprinkling method of Duddington (1955).

Assessing the mycelium growth of the nematophagous fungi

Disks (5 mm in diameter) of the fungi *M. sinense* and *P. chlamydosporia*, obtained from pure cultivation in 2% CMA culture medium, were transferred separately to the centre of 9-cm diameter Petri dishes containing 20 mL of 2% water agar medium (2% WA) (Kasvi®) at pH 7. The plates were kept in a BOD (biochemical oxygen demand) incubator at temperatures of 15, 20, 25, 30, 35 and 40°C in the dark. This same procedure was performed using 2% potato dextrose agar medium (2% PDA) (kasvi®) and 2% corn meal agar medium (2% CMA) (Sigma-Aldrich®) at pH 7. These treatments consisted of a 2 x 3 factorial arrangement, with two isolates of nematophagous fungi and three culture media. The experimental design was completely randomised with six replications, with each replication consisting of one Petri dish.

Observations were started 24 hours after starting the experiment. The mycelial growth of the isolates was determined by measuring the diameter of the colonies at 24-hour intervals, using a millimetre ruler, taking two measurements in perpendicular directions on each plate, as shown in Figure 1. For 6 days, measurements of mycelial growth were carried out until the growth of the colony of one of the isolates occupied the entire surface of the medium (Lilly and Barnett 1951).

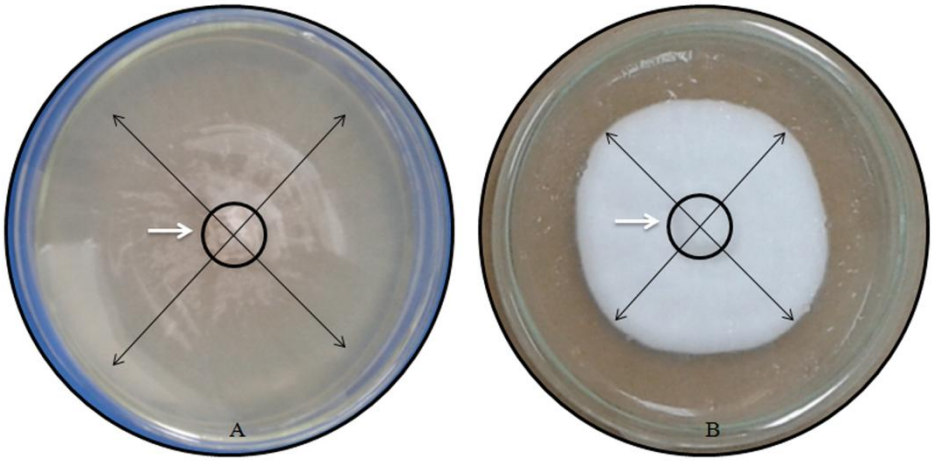

Figure 1: Petri dishes used in the growth bioassay of the fungi *Monacrosporium sinense* (A) and *Pochonia chlamydosporia* (B). White arrow indicates the edge of initial inoculum. Black arrows represent measurements of fungi mycelium radial growth.

The mycelial growth rate index was calculated according to the formula described by Oliveira (1991):

$$MGRI = \Sigma (D - Da)/N,$$

where MGRI (cm/day) is the mycelial growth rate index; D is the current average colony diameter; Da is the mean diameter of the colony from the previous day; N is the number of days after inoculation. After the measurements, in case a certain temperature totally inhibited fungal growth on the 6th day, these plates were transferred to a BOD at 25°C and continuously observed daily for another 9 days, resulting, for this specific case, in 15 observations. Over these 9 days, no measurements were made.

In parallel, 5-mm diameter disks of VC4 and SF53 strains were inoculated in the centre of Petri dishes containing 2% BDA medium to analyse the effect of pH on growth. The pH values of the 2% BDA medium were adjusted from 4 to 10 using an acidometer and 3.0 M NaOH solution. The Petri dishes were incubated for 6 days, at 25°C, in the dark in a BOD chamber. The mycelial growth rate index was evaluated as described above in the temperature test, and six repetitions were performed for each fungal strain at each pH value.

The mean values of the MGRI for each temperature and pH condition were submitted to the Kruskal-Wallis nonparametric statistical test at a significance level of 5%. The analyses were performed using the IBM SPSS Statistics 2.0 software.

Electron and optical microscopy of the nematophagous fungi and interactions with bovine gastrointestinal nematodes

Images of the fungal mycelia, their structures and interactions with bovine gastrointestinal nematode larvae were obtained under optical and scanning electron microscopes (SEM). The preparations for SEM followed the technique proposed by Nordbring-Hetz (1983), with modifications. Cultures of SF53 and VC4 isolates were separately plated on dialysis membranes in Petri dishes containing 2% WA medium and incubated in the dark at 25°C for 21 days. Subsequently, approximately 500 infective larvae (L3) of bovine gastrointestinal parasites previously obtained by coprocultures were dripped onto the cultures of each fungus grown on a dialysis membrane surface. After 72 hours of fungi-nematode interactions, cultures were fixed on plates with 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4, for 72 h. Subsequently, the plates were washed six times in the same buffer. With the aid of a scalpel, dialysis membrane flaps were cut and collected with fine-tipped forceps and then dehydrated through an alcohol serial passage (30, 50, 60, 70, 95 and 100%). Then, the samples were dried in a BALZERS® critical point dryer using carbon dioxide, covered with gold in a metalliser and electronmicrographed in a LEO scanning electron microscope at 10–15 kV at the Center for Electron Microscopy and Microanalysis at the Federal University of Vicosa.

Results

Figures 2 and 3 illustrate *Monacrosporium sinense* morphology with subglobose conidia with one to three septa 23–30 x 17–25 µm, spherical chlamydospores with thick-walled and intercalated along the hyphae and adhesive networks capable of trapping nematode larvae.

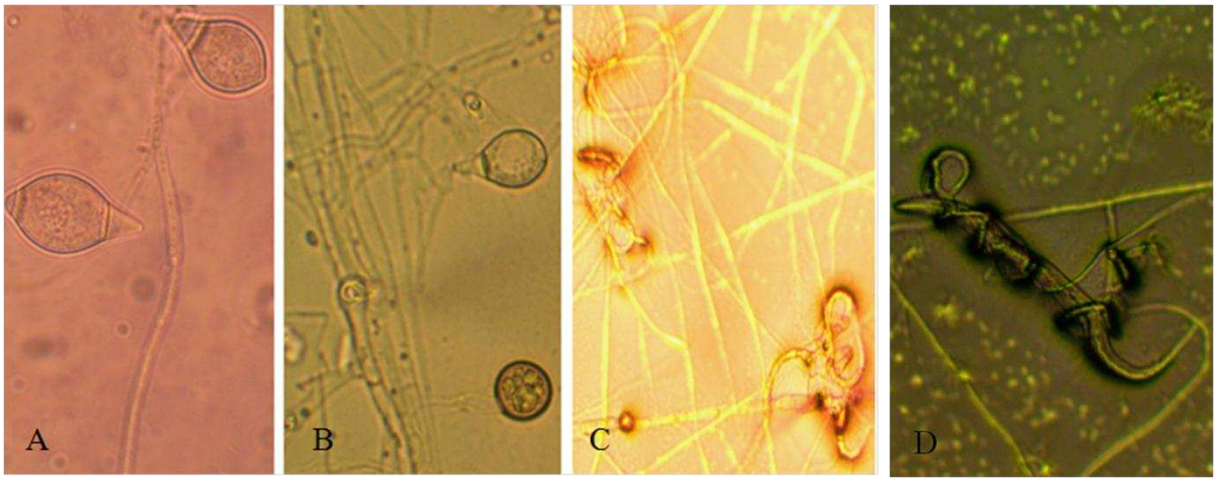

Figure 2: Images of the fungus *Monacrosporium sinense* under light microscope. (A) conidia with 2 and 3 septa respectively and conidiophore at 40X magnification, (B) conidia (top) and chlamydospore (bottom) at 40X magnification, (C) adhesive network trap at 10X magnification, (D) trapped bovine gastrointestinal nematode larvae at 10X magnification.

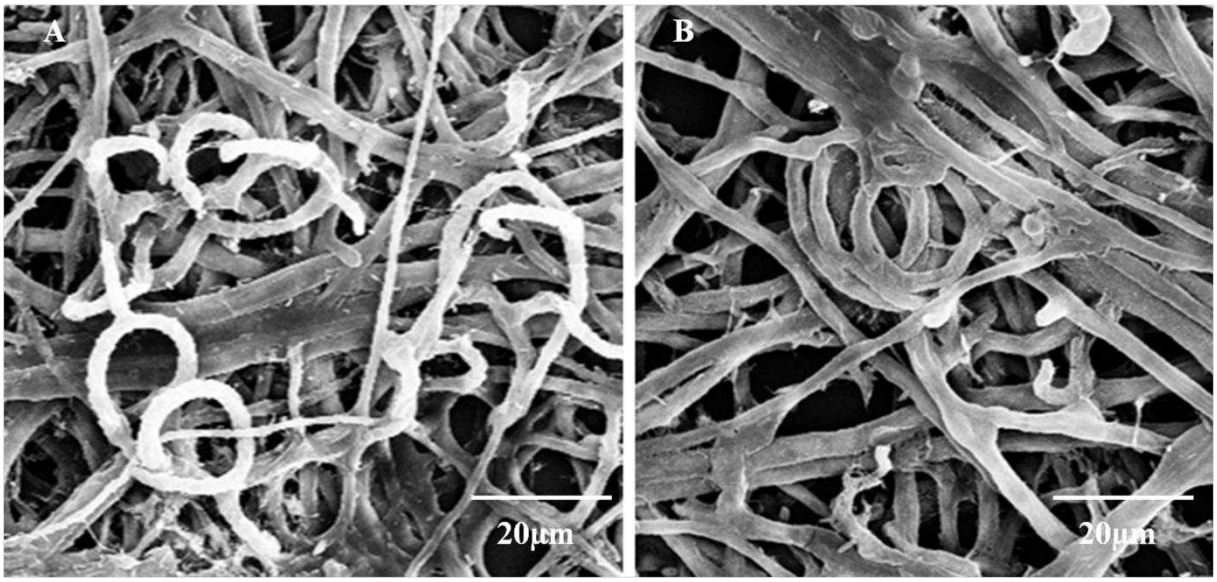

Figure 3: Images of the fungus *Monacrosporium sinense* mycelium and adhesive network under electron microscope.

Figures 4 and 5 show conidia and chlamydospore of *Pochonia chlamydosporia* and its predation ability on nematode larvae. As shown in Table 1, *M. sinense* had the numerically highest growth rate at 30°C, regardless of the culture medium used, and the growth rate at 15°C was significantly lowest ($p \leq 0.05$) in 2% WA medium. The growth rate index of *P. chlamydosporia* was higher in 2% WA at 20°C and in 2% PDA and 2% CMA at 30°C. There were significant differences in MGRI values of *P. chlamydosporia* between 15 and 25°C in 2% WA and between 15 and 30°C in 2% PDA and 2% CMA ($p \leq 0.05$).

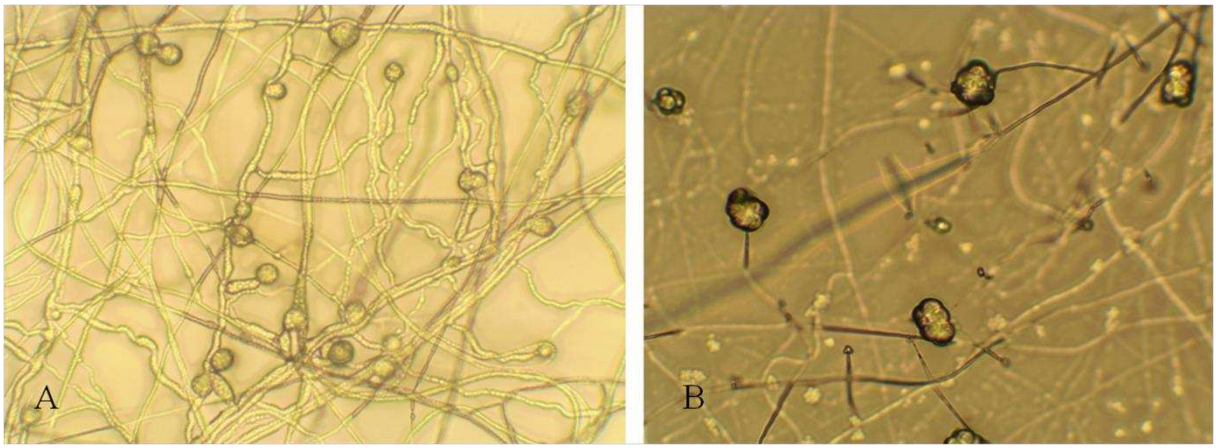

Figure 4: Images of the fungus *Pochonia chlamydosporia* under light microscope. (A) conidia and mycelium at 10X magnification; (B) chlamydospores at 40X magnification.

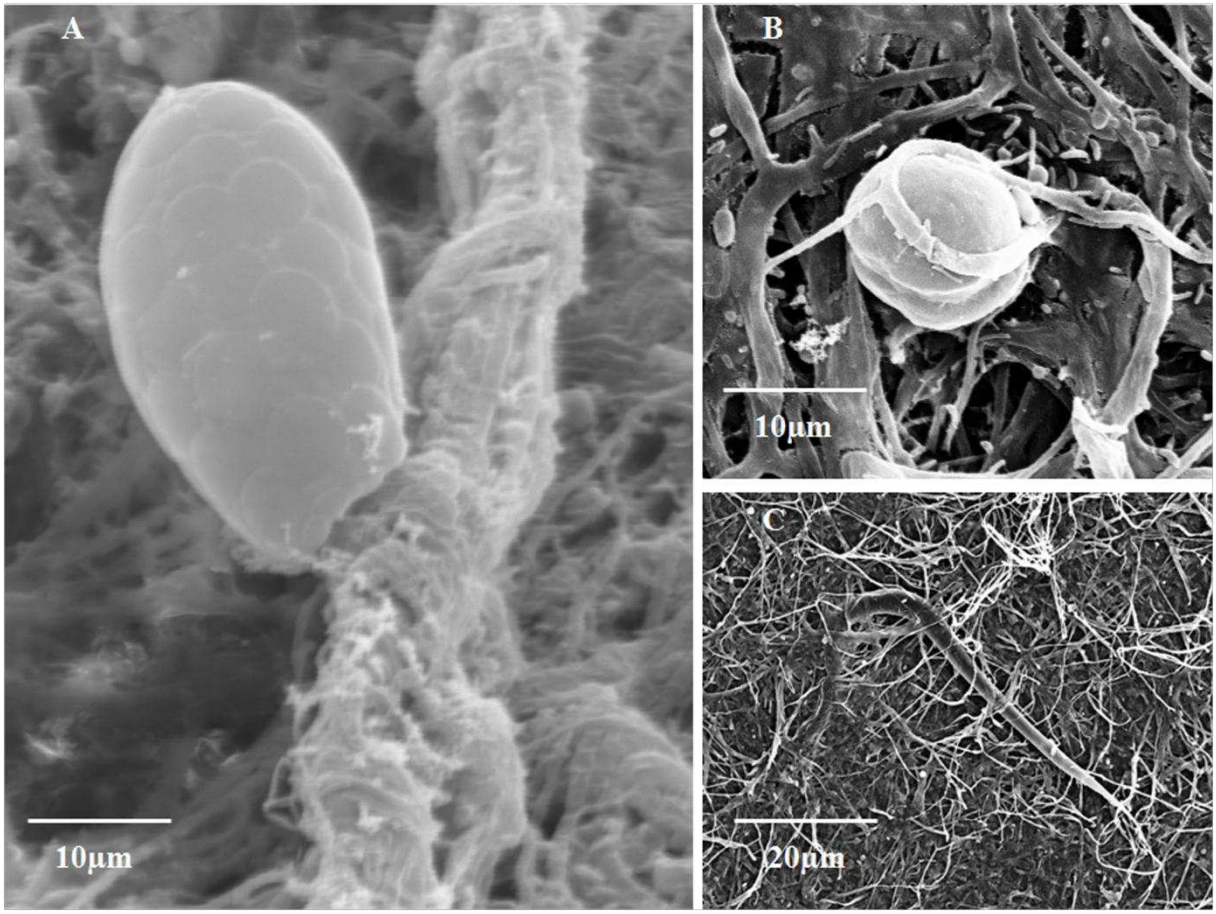

Figure 5: Images of the fungus *Pochonia chlamydosporia* under electron microscope. (A) conidia; (B) chlamydospore; (C) bovine gastrointestinal larvae trapped in the mycelium.

Table 1: Mean values and standard deviations (between parentheses) of mycelial growth rate index (MGRI) of the fungi *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4) grown in plates containing 2% water agar medium (2% WA), 2% potato dextrose agar medium (2% PDA) and 2% corn meal agar medium (2% CMA) at temperatures of 15, 20, 25, 30, 35 and 40°C, for six days.

	Monacrosporium sinense (SF53)			*Pochonia chlamydosporia* (VC4)		
	2% WA	2% PDA	2% CMA	2% WA	2% PDA	2% CMA
T(°C)	MGRI	MGRI	MGRI	MGRI	MGRI	MGRI
15	0.28^{a} (0.06)	0.17^{a} (0.08)	0.35^{a} (0.07)	0.12^{a} (0.11)	0.14^{a} (0.15)	0.12^{a} (0.09)
20	0.61^{b} (0.26)	0.49^{ac} (0.55)	0.61^{a} (0,32)	0.25^{ab} (0.16)	0.19^{ab} (0.16)	0.27^{ab} (0.20)
25	0.73^{b} (0.34)	$0,57^{bc}$ (0.21)	0.77^{a} (0.50)	0.23^{b} (0.07)	0.24^{ab} (0.08)	0.27^{b} (0.10)
30	0.84^{b} (0.44)	0.74^{b} (0.21)	0.92^{a} (0.65)	0.20^{ab} (0.09)	0.33^{b} (0.12)	0.30^{b} (0.10)
35	0	0	0	0	0	0
40	0	0	0	0	0	0

*Same lowercase letters in the same column indicate that there is no significant difference ($p \geq 0.05$) between the data. MGRI: cm/day.

The two fungi grew at all tested pH levels, as presented in Table 2.

Table 2: Mean values and standard deviations (between parentheses) of the mycelial growth rate index (MGRI) of *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4), grown in 2% potato dextrose agar medium (2% PDA), at pH values from 4 to 10.

pH	SF53	VC4
4	0.33^{a} (0.25)	0.24^{a} (0.22)
5	0.54^{ac} (0.46)	0.28^{ab} (0.28)
6	0.58^{ac} (0.29)	0.35^{ab} (0.26)
7	0.68^{bc} (0.34)	0.36^{ab} (0.30)
8	0.68^{bc} (0.32)	0.23^{ab} (0.06)
9	0.68^{bc} (0.30)	0.22^{ab} (0.06)
10	0.61^{ac} (0.34)	0.31^{b} (0.10)

*Different letters in the same column indicate a difference between the data ($p \leq 0.05$). SF53: *Monacrosporium sinense*; VC4: *Pochonia chlamydosporia*.

Discussion

Both fungi showed high conidium and chlamydospore production, which are important structures to facilitate their survival and establishment in the environment. The predator fungus *M. sinense* modified its hyphae in adhesive networks capable of trapping the nematode, as reported by Campos *et al*. (2007). Nematode larvae held by *P. chlamydosporia* mycelium were seen by scanning electron microscopy, confirming its ability to colonise the nematode larvae stage and to penetrate the larvae by mechanical and enzymatic actions, despite belonging to the "ovicidal" group. Recently, the possibility of obtaining high, efficient

parasite control by the union of these distinct fungal isolates and compatible predatory abilities has been demonstrated (Oliveira *et al*. 2021a), making it important to consider *P. chlamydosporia* larvae predation. In this sense, Vieira *et al*. (2019) showed a reduction of 92.67% on infective bovine larvae when associating *Pochonia chlamydosporia* with *Arthrobotrys cladodes*. These studies open possibilities for new commercial formulations. In the last 5 years, commercial formulations containing *Duddingtonia flagrans* have started to become commercially available in Brazil (Bioverm®), Australia and New Zealand (BioWorma®), and these products are already used in animal feed (Araújo *et al*. 2021). Thus, this is an opportunity to explore the wide and promising field of research in parasitic biological control to find and analyse other factors that can ensure an even more successful performance of these agents (Vieira *et al*. 2020; Araújo *et al*. 2021; Oliveira *et al*. 2021a).

In-vitro tests have showed that temperature was a limiting factor in the growth of nematophagous fungi *M. sinense* and *P. chlamydosporia*. No growth of fungal isolates was observed at 35 or 40°C over the 6 days. However, interestingly, the mycelial growth of both strains inhibited at 35°C started after these plates were incubated again at 25°C. This result shows that the initial temperature for the mycelial growth of fungi is of essential importance in their establishment in the environment.

In the present study, the highest growth rate of *M. sinense* was similar to observations reported by Xue *et al*. (2018), who analysed the temperature influence on the growth of *Arthrobotrys sinense* from China and observed mycelial growth between 11 and 35°C, with maximum levels at 30°C. These findings allow predicting in which microclimate it will be possible to keep the fungus abundant in the environment and to facilitate nematode infective form predation. The MGRI results for *P. chlamydosporia* were similar compared to those reported by Vieira *et al*. (2020), who showed that *P. chlamydosporia* colony growth was higher at intermediate temperatures (20, 25 and 30°C) than at temperature extremes of 15 and 35°C in PDA medium over 10 days. In addition, understanding the temperature influence is crucial for storing process improvement of these microorganisms.

In the current assay, temperature had a higher influence on the extensive hyphae system formation than the culture media composition. This observation supports the statement about the capacity of these microorganisms to adapt to different environmental conditions, allowing them to be found dispersed in a variety of ecosystems (Braga and Araújo, 2014; Zhang *et al*. 2014), which increase their potential use. Despite the interference observed in the present study in some temperature conditions, these fungal isolates are promising agents in nematode control. The combination of *M. sinense* and *P.chlamydosporia* demonstrated a 98.90%

reduction in the number of bovine nematode infective larvae under *in-vitro* conditions and at 25°C (Oliveira *et al*. 2021a). Surely, rapid colonisation in the environment where the fungi will be inserted is an important step to provide them competitive advantage, and Vieira *et al*. (2020) emphasised that fungal strains which demonstrate greater resistance to the adversities found in the environment would be most suitable for the use in biological nematode control strategies.

Similar to the temperature, the pH can affect fungal metabolism, resulting in changes in their growth. The observations made here are important to show that animal gastrointestinal tract pH variations will not be harmful to fungi. Resistance to passage through the gastrointestinal tract is an important characteristic in fungi to be used in biological control since the use of oral formulations in animals is the most practical way for these fungi to be dispersed and to colonise the faecal pats to act on nematode infective stages. Interestingly, ruminant saliva has an alkaline pH of around 8.1, whereas the ruminal pH of grazing cattle remains close to neutrality (5.5 and 7) (Oliveira *et al*. 2019), corroborating with pH variations found in our study. However, characterising the environmental conditions favouring the growth capacity at different pH values appears necessary for implementing suitable conditions in the laboratory and commercial production of nematophagous fungi. The species *P. chlamydosporia* showed a higher growth rate at pH 7, and there were significant differences in MGRI values only between pH 4 and 10 ($p \leq 0.05$). The species *M. sinense* showed highest growth rates in a pH range from 7 to 9 and lowest values at pH 4. In addition, pH evaluation in the present study seemed realistic regarding the natural environment where fungi will be inoculated to establish growth. Xavier *et al*. (2016) reported that beef cattle kept in a pasture of *Brachiaria brizantha* and receiving only protein salt had a faecal pH of 7.4. This leads us to infer that fungal isolates, after passing through the animal gastrointestinal tract, will find favourable pH conditions for growth on faecal pats and, consequently, will be able to prey upon nematodes.

Although this study focused on macroscopic fungal growth assessment, the limited use of nematophagous fungi for controlling animal gastrointestinal nematodes seems partly due to the lack of the elucidation of chemical, physical and biological factors that affect the development of these microorganisms in the environment. Vieira *et al*. (2020) emphasised that bioassays under laboratory conditions are an important step in the selection of potential candidates to be used in the control of gastrointestinal parasitic nematodes.

Although *M. sinense* and *P. chlamydosporia* showed synergistic action, suggesting that the joint application of fungi increases the effectiveness of the biological control of bovine

infectious larvae (Oliveira *et al*. 2021a), it is relevant to obtain a better understanding of factors that can influence their growth to enhance the availability of promising fungi. Grazing systems are generally complex, with several biotic and abiotic factors, and fungi can therefore show variations in growth and predation percentages. In this study, temperature was a decisive variable in the mycelial distribution in the culture medium, and consequently, the study of each strain must be specific. However, field evaluations with experimental formulations by a sodium alginate matrix with different fungal isolates (Mendoza-de-Gives *et al*. 2018; Oliveira *et al*. 2018; Vieira *et al*. 2020; Voinot *et al*. 2020) and commercial formulations containing structures of the fungus *Duddingtonia flagrans* (Braga *et al*. 2020; Oliveira *et al*. 2021b) demonstrated excellent results in reducing the infective forms of gastrointestinal nematodes, but scientific data are still scarce. Furthermore, Ocampo-Gutiérrez *et al*. (2021) emphasised the need for future work with nematophagous fungal mycelia to elucidate the presence of intracellular products that may be crucial in their nematocidal activity.

Defining the physiological requirements of the fungi *M. sinense* and *P. chlamydosporia* is essential to ensure their success as biocontrolers, and *in-vitro* studies can facilitate the exploration of these agents. Further studies should focus on understanding other fundamental aspects about nematophagous fungal ecology of biotechnological interest for controlling gastrointestinal nematodes in animals.

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Author Contribution

Isabela and Jackson Victor conceived and designed the study. Isabela conducted data gathering. Ítalo performed statistical analyses. Isabela, Ítalo, Samuel, Artur, Adolfo, Cristiana, Pedro and Jackson Victor wrote the article.

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Conflicts of Interest

The authors declare there are no conflicts of interest.

Ethical Standards

'Not applicable' here.

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7. CAPÍTULO 3

Monacrosporium sinense and *Pochonia chlamydosporia* for the biological control of bovine infective larvae in *Brachiaria brizantha* pasture

Monacrosporium sinense and *Pochonia chlamydosporia* for the biological control of bovine infective larvae in *Brachiaria brizantha* pasture

Abstract

Ecologically correct solutions are necessary to minimize the use of anthelmintics in the control of cattle parasitic diseases. This study evaluated the effects of oral administration pellets containing the fungal combination *Monacrosporium sinense* and *Pochonia chlamydosporia* in the biological control of infective larvae (L3) of parasitic gastrointestinal nematodes of cattle grazed in pastures. Twelve crossbred calves (Holstein x Zebu), seven to nine years old, after dewormed, were randomly divided into two groups (group treated with the combination of fungi and control group) and placed in separate paddocks of *Brachiaria brizantha* that were naturally infested with L3. Feces samples were collected from the animals to determine the parasitic load and pasture samples were collected from the soil to determine the L3 infestation. The recovery of L3 from the pasture of the treated group was 91.7% and 86.4% less than in the pasture of the control group, respectively for samples collected at 20 cm and 40 cm from the faecal pats. The values of eggs per gram of feces in the months of May 2019 and January 2020 were lower ($p \leq 0.05$) in the treated group with the fungal combination. The combined administration of *M. sinense* and *P. chlamydosporia* reduced the L3 infestation on pastures and in animal parasitic load, making it a tool for controlling bovine gastrointestinal parasitic nematodes.

Keywords: biocontrol, cattle, environment, nematophagous fungi, parasite, worms

1. Introduction

Pastures use in cattle grazing favors Brazilian livestock production, since pastures are associated with the practical and economic management of animals (Dias-Filho, 2016). Brazil has the largest commercial cattle herd in the world (IBGE, 2020), with great potential for milk and meat production (ABIEC, 2020; IBGE, 2020). However, production is severely hampered by helminths (Grisi *et al*. 2014). There are several species of gastrointestinal parasitic helminths in regions with a tropical and subtropical climate, where climatic conditions favour their multiplication. The most prevalent genera causing parasites in Brazilian cattle are *Haemonchus*, *Cooperia*, *Oesophagostomum* and *Trichostrongylus* (Oliveira *et al*. 2017).

The impacts of helminths in animal production include delayed body development and reduced productive and reproductive performance, favouring the occurrence of various

diseases and death (Lambertz *et al*. 2019). The control of gastrointestinal nematode infections is mostly based on broad spectrum anthelmintics use (Kaplan, 2020). The largest revenue of Brazilian veterinary medicine industry is due to the trade antiparasitic products (SINDAN, 2017), which are highly demanded in ruminants creation. The use of chemical compounds only eliminates the parasites adult stages that reside in the animal's gastrointestinal tract. The free-living stages, on the other hand, remain viable for weeks or even months on pastures. These immature stages hatch and develop in the environment and they are again ingested by animals, continuing their parasitic life cycle (Voinot *et al*. 2020). In addition of this limiting factor regarding the use of chemical compounds, anthelmintic resistance is severe worldwide (Kaplan, 2020). At the same time, one of the greatest challenges in livestock production will be ensuring productivity to feed the high market demand without increasing the degradation of the environment (ABCBIO, 2015).

Thus, bioproducts for controlling gastrointestinal nematodes are a desirable tool to compose nematodes management of grazing cattle (Araújo *et al*. 2021). Given the excellent results obtained in studies using several genera of nematophagous fungi (Mendonza-de-Gives *et al*. 2018; Oliveira *et al*. 2018; Vieira *et al*. 2020; Voinot *et al*. 2020), biological control with nematophagous fungi stands out as a good tool for parasite management (Araújo *et al*. 2021; Canhão-Dias *et al*. 2020; Mendoza-de-Gives *et al*. 2022) by reducing the use of chemical anthelmintics that impact the environment. Nematophagous fungi are naturally present in the environment and are cosmopolitan in their distribution (Zhang and Hyde, 2014). The use of these biological agents by oral administration to the animals for control helminths is viable option (Araújo *et al*. 2021; Mendoza-de-Gives *et al*. 2022), and works to select ideal fungal isolates has encouraged researchers to provide better efficient commercial formulations.

The fungus *Pochonia chlamydosporia* parasitises eggs and female nematodes (Dallemole-Giaretta *et al*. 2013; Vieira *et al*. 2019) and belongs to the group to the nematophagous fungal group known as "ovicidal fungi" (Araújo *et al*. 2021). The species *Monacrosporium sinense* predates nematode larvae through adhesive networks (Campos *et al*. 2007). In a previous study, the combination of *M. sinense* and *P.chlamydosporia* demonstrated a 98.90% reduction in the number of bovine nematode infective larvae under *in vitro* conditions and at 25°C (Oliveira *et al*. 2021). This work aimed to evaluate the effects of the inclusion of *Monacrosporium sinense* and *Pochonia chlamydosporia* in pellets of sodium alginate on pasture infestation and parasitic load in animals.

2. Material and methods

Nematophagous fungi *Monacrosporium sinense* (SF53 isolate) and *Pochonia chlamydosporia* (VC4 isolate) are part of the collection of the Parasitology Laboratory of the Veterinary Department of the Federal University of Viçosa, Minas Gerais, where they were kept at 4 °C under a light in test tubes containing 2% corn meal agar (2% CMA) medium. The fungus was inoculated in Petri dishes (9 cm diameter) containing 2% water agar medium (2% WA). The fungus was allowed to grow for seven days. For induction of fungal mycelium formation, approximately 5 mm agar fragments containing mycelium and fungal spores were transferred to 250 ml Erlenmeyer flasks containing 150 ml of liquid medium GPY (glucose, soy peptone and yeast extract) and pH 6.5. Contents were stirred at 120 rpm, in the dark conditions at 26 °C for 21 days. After this period, the mycelium was removed for manufacturing the pellets, which were incorporated in sodium alginate matrix according to the technique described by Walker and Connick (1983) modified by Lackey *et al*. (1993).

The experiment was conducted on a farm located in the municipality of Abre Campo, state of Minas Gerais, southeastern Brazil, latitude 20º 18 '04 "S, longitude 42º 28' 39" W.

Twelve female, crossbred Holstein x Zebu bovines, seven to nine years old, with a mean body weight of 150 kg were previously treated with the albendazole anthelmintic suspension (CALBENDAZOLE®) orally, an single dosage of 1 mL / 20kg body weight. Twenty-one days after the anthelmintic treatment, the animals were confirmed to have zero eggs per gram of feces and randomly divided into two groups with six animals each and placed in two paddocks of *Brachiaria brizantha*, naturally infested with helminth larvae, having previously been used for grazing by young and adult animals. In the treatment group, each bovine was treated with 1 g of pellets / 10 kg of body weight (0.1 g of *M. sinense* + 0.1 g of *P. chlamydosporia* / 10 kg body weight) containing the fungal combination *M. sinense* and *P. chlamydosporia* administered twice a week with wheat bran. In the control group, each animal received 1 g of pellets without fungal mycelium / 10 kg of body weight with wheat bran.

The experiment lasted for 10 months, during which samples of feces and pasture vegetation were collected. The methodology was consistent with the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) following the guidelines for assessing the effectiveness of anthelmintics in cattle and sheep described by Powers *et al*. (1982) and the second edition of the guidelines by Wood *et al*. (1995). This experimental trial strictly followed all the procedures recommended by the rules of conduct

for the use of animals and certified by the Ethics Committee on the Use of Animals (CEUA/UFV), under reference no. 91/2018.

Every 15 days after the animals had been introduced onto the pastures, feces samples were collected from all animals in each group directly from the rectum. Eggs counts per gram of feces (EPG) were determined using the method of Gordon and Whithlock (1939), with modifications by Lima (1989). This technique is performed as follows: weigh 2 grams of feces; place the feces in a glass beaker adding 20ml of saturated sodium chloride solution; the feces are crushed and homogenized with a glass rod and sieved into another beaker. Pass through the sieve a solution containing 14.5mL of saturated sodium chloride solution and 14.5mL of water. Homogenize the faecal solution and fill the McMaster chamber with the solution. The eggs counting should be performed under a light microscope using a 10X objective. Each egg found must be multiplied by 100. In parallel, to complement the EPG, coprocultures were produced using 20 g of feces mixed with vermiculite and were placed in Biochemical Oxygen Demand (BOD) incubator at 26°C for 15 days to obtain infective larvae (L3). At the end of this period, the L3 were recovered from the coprocultures by soaking in water at 42-45°C in a Baermann apparatus and collected them in hemolysis tubes after 12 hours of decantation. The infective larvae genus was later identified according to Keith (1953).

Every 15 days from the start of the experiment, two *Brachiaria brizantha* pasture samples (0–20 cm and 20–40 cm away from the faecal pats) were collected from the paddocks of the treated and control groups from six different points, according to Raynaud and Gruner (1982). Samples of 500 g of pasture were used to recover the infective larvae (L3) following the methodology described by Lima (1989). The L3 were recovered by soaking pasture samples in water at 42-45°C in a Baermann apparatus and collecting them in graduated beaker after 12 hours of decantation. The sediment was examined under an optical microscope and the larvae were counted and identified according to the criteria established by Keith (1953). The 500 g samples of pasture that were used for this method were placed in an oven at 100°C to obtain dry matter. The data obtained were transformed into the number of larvae per kilogram of dry matter.

The animals were weighed monthly to determine the mean daily weight gain through the formula: weight gain = (body weight in the current month - body weight in the previous month)/number of days elapsed between weighings.

The climatic data referring to the average monthly minimum and maximum temperatures and monthly rainfall were obtained from the Agrometeorological Monitoring System (Agritempo), Brazil.

The averages of the egg counts per gram of feces (EPG), the nematodes that were recovered in the coprocultures and the L3 from the pasture samples, the weight of the animals and the climatic data during the 10 months of the experiment were converted to monthly averages.

Subjected to Levene's test, the OPG data were not normally distributed. Therefore, the independent monthly data of each group were compared using the non-parametric Mann-Whitney "U" test, at a significance level of 5%.

Data from coprocultures were normally distributed (Levene's test: $p \geq 0.05$) and were submitted to Analysis of Variance (ANOVA) and Tukey's "f" test, at a significance level of 5%.

Weight gain data were normally distributed (Levene test: $p \geq 0.05$). For the same month of the experiment, the independent monthly data of each group were compared using the Student's "t" test, at a significance level of 5%.

The larvae recovery data in the pasture did not show a normal distribution (Levene test: $p \leq 0.05$). Thus, the data of each group, throughout the experimental period, were compared using the non-parametric Kruskal-Wallis test, at a significance level of 5%. All analyses were performed using the software IBM SPSS Statistics 2.0.

3. Results

Figure 1 shows the monthly mean number of eggs per gram of feces (EPG) of the treated group and the control group during the period from April 2019 to January 2020. In the first month of treatment, the low number of EPG was the result of anthelmintic treatment administered to animals prior to the beginning of the experiment. The EPG values in the months of May 2019 and January 2020 were lower ($p \leq 0.05$) in the treated group. The mean EPG values of animals in the control group in October, November and December 2019 were numerically higher than the average EPG values of the treated group.

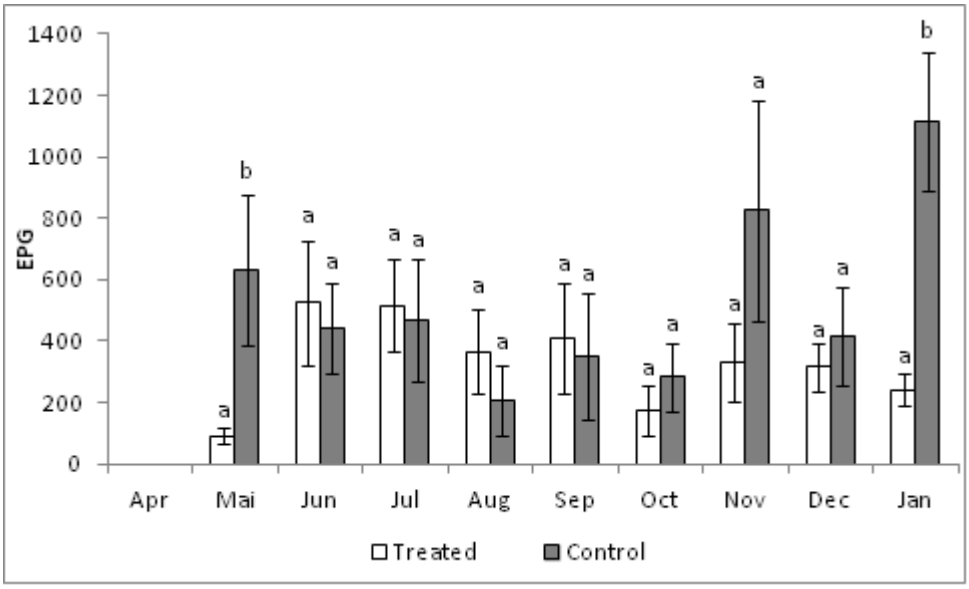

Figure 1: Monthly means and standard error of the number of eggs per gram of feces (EPG) in the group treated with the fungal combination *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* and in the control group during the period from April 2019 to January 2020, in Abre Campo, Minas Gerais, Brazil. Same letters in the same month indicate that there is no significant difference ($p \leq 0.05$) between the data.

Table 1 shows the percentage values of the L3 genera recovered from coprocultures of the feces of the animals in the group treated with the fungal combination *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4) and the control group. There was no significant difference ($p \leq 0.05$) between the data.

Table 1. Mean values of the percentages of the genera *Haemonchus* (Haem), *Cooperia* (Coop) and *Oesophagostomum* (Oeso) infective larvae recovered from coprocultures of groups of animals treated with the fungal combination of *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4) and the control group during the period from April 2019 to January 2020, in Abre Campo, Minas Gerais, Brazil.

Month	Treated			Control		
	Haem	*Coop*	*Oeso*	*Haem*	*Coop*	*Oeso*
Apr	63.2	34.1	2.8	51.7	48.3	0.0
Mai	64.7	35.1	0.3	59.0	40.8	0.2
Jun	61.9	37.2	0.9	54.3	39.3	6.5
Jul	50.3	44.2	5.6	52.5	27.1	20.4
Aug	66.2	27.2	6.6	70.0	14.0	16.0
Set	57.9	35.1	7.0	53.3	24.4	22.3
Oct	38.2	27.5	34.3	50.9	30.3	18.8
Nov	41.5	43.8	14.7	45.1	41.0	13.9
Dec	57.3	15.4	27.3	41.7	36.5	21.8
Jan	44.1	22.7	33.3	43.8	37.9	18.3
Mean	54.5 a	32.2 b	13.3 c	52.2 a	34.0 b	13.8 c
MSE	3.3	2.9	4.2	2.6	3.2	2.7

Different letters on the same line indicate a significant difference between the data ($p \leq 0.05$).
MSE: Mean standard error

Haemonchus sp. was the most prevalent followed by *Cooperia* sp. and *Oesophagostomum* sp. in both groups.

The means of larvae recovered from pastures differed significantly ($p \leq 0.05$) between the sample from the control group at a distance of 20 cm and treated sample at a distance of 20-40 cm, as shown in Table 2.

Table 2. Mean values of infective larvae recovered per kilogram of dry matter in the group treated with the fungal combination of *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4) and in the control group during the period from April 2019 to January 2020, in Abre Campo, Minas Gerais, Brazil. Different letters on the same line indicate a significant difference between the data ($p \leq 0.05$).

	Larvae / Kg Dry Matter (0-20 cm)		Larvae / Kg Dry Matter (20-40 cm)	
Month	Treated	Control	Treated	Control
Apr	73.33	98.04	10.15	26.33
Mai	4.19	126.11	0.00	31.78
Jun	30.19	685.31	16.91	294.59
Jul	7.69	139.33	2.50	33.80
Aug	48.97	34.74	0.00	0.00
Sep	16.62	159.36	5.34	6.06
Oct	90.50	3152.94	31.02	458.56
Nov	424.25	4113.04	248.06	1205.30
Dec	7.80	56.27	4.39	33.15
Jan	73.58	806.13	45.18	592.97
Mean	77.7 ab	937.1 a	36.4 b	268.3 ab
MSE	39.8	462.7	24.0	124.1

Different letters on the same line indicate a significant difference between the data ($p \leq 0.05$). MSE: Mean standard error.

Figure 2 shows the weight gain (kg/day) in the group treated with the fungal combination *M. sinense* (SF53) and *P. chlamydosporia* (VC4) and in the control group during the experimental period. There was a significant difference ($p \leq 0.05$) between the animals' weight gain in the groups studied in January 2020. The body weight gain was greater ($p \leq 0.05$) in the treated group than in the control group in January 2020.

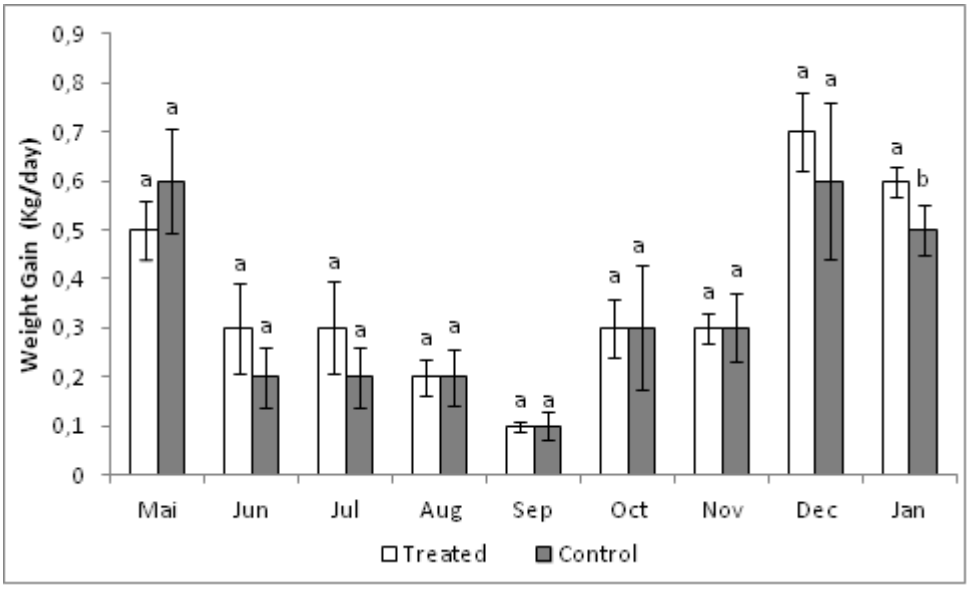

Figure 2: Mean weight gain (kg/day) and standard error (bars) in the group treated with the fungal combination of *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4) and in the control group during the period from April 2019 to January 2020, in Abre Campo, Minas Gerais, Brazil. Same letters in the same month indicate that there is no significant difference ($p \leq 0.05$) between the data.

Table 3 presents the minimum, mean and maximum monthly temperatures, as well as the monthly rainfall during the experimental period in the city of Abre Campo, Minas Gerais, Brazil. The lowest temperatures recorded occurred in the months of July and August 2019. Mean monthly temperatures oscillated between 18.24°C in July 2019 and 25°C in December 2019. The highest rainfall rates were recorded in August and September 2019 and January 2020.

Table 3. Minimum Temperature (T Min), Average (T Mean), Maximum (T Max) and Precipitation (mm^3/month) values from April 2019 to January 2020 in Abre Campo, Minas Gerais, Brazil.

Month	T Min	T Mean	T Max	Precipitation (mm^3)
Apr	16.00	23.02	30.00	21.18
Ma	14.00	21.97	30.00	50.39
Jun	10.00	19.80	29.00	24.49
Jul	7.00	18.24	29.00	9.43
Aug	7.00	18.92	29.00	42.46
Sep	13.00	21.97	34.00	55.07
Oct	16.00	22.97	34.00	2.86
Nov	18.00	23.38	31.00	12.84
Dec	20.00	25.00	30.00	0.01
Jan	17.00	23.73	34.00	295.67

4. Discussion

Due to the need to adapt ruminant rearing systems to low chemical drugs dependence for nematode control, the results of this research indicates that the use of nematophagous fungal association represents a convenient tool for controlling gastrointestinal nematodes on pasture.

The anthelmintic administered to the animals reflected in eggs absence in animal feces at the beginning experiment. The deworming eliminates the parasites adult phases that reside in the animals' gastrointestinal tract (Kaplan, 2020) which interfered in the amount of helminth eggs eliminated in the feces. However, from the moment that the two groups of animals were placed in *Brachiaria brizantha* pastures, the animals were naturally exposed to free-living stages of gastrointestinal parasites, allowing reinfection (Figure 1). The most of bovine parasitic worms is found on pastures (Voinot *et al*. 2020), which is why restricted control with antiparasitic drugs is not satisfactory.

The EPG counting does not reflect exact number of adult helminths in the animals, but it is a method for evaluating parasitic load and it is fundamental in making decision on health management program in cattle. Fungal combination use *M. sinense* (SF53) and *P. chlamydosporia* (VC4) significantly reduced the EPG count of the treated group compared to

the control group in some experimental periods. Vieira *et al*. (2020) administered the fungal combination *P. chlamydosporia* and *Arthrobotrys cladodes* to cattle and found that the EPG values of the treated animals were 96.7% lower than the control group. Both results emphasize that, when it is intended to prevent the action of nematodes in animals, it is important to afford treatments that act in the infectious forms on pasture. In contrast, Delgado *et al*. (2009) demonstrated that it is minimal the ranchers perception in relation by the worms importance in the herd and that anthelmintics are widely used and in the most cases without using any technical criteria.

In the present study, we found that the parasitic load of animals treated with fungi was lower than the control group from October to December 2019 (Figure 1). It is important to consider that animals with low EPG values will require less intervention by anthelmintic treatments, corroborating observations made by Silva *et al*. (2010) who used the fungus *Arthrobotrys robusta* to control gastrointestinal parasites in sheep. A reduction in the use of anthelmintics can also reduce the appearance of nematodes resistant to these products and it is essential to extend the useful life of these synthetic products (Shalaby, 2013).

The variation in the EPG illustrates the effect of the environment on animal parasitic load. Regarding the severity of the infection, the animals in the group treated with the fungal association had a mild (<200 EPG) to moderate (200 to 600 EPG) degree of infection, whereas in animals in the control group infection ranged from mild to heavy (> 700 EPG) (Ueno and Gonçalves, 1998). The low EPG count of the treated group can be attributed to the presence of fungi in the faecal pats, which reduces the number of larvae ingested by the animals. Campos *et al*. (2007) administered pellets containing the fungi *M. sinense* to cattle raised in the field and observed a 58% reduction in the number of L3 in the pasture of the treated animals, which was reflected in the 79% reduction of the EPG values of these same animals. Such the larvae presented in the pasture influences the severity infection in the animals, so it is relevant to use nematophagous fungi that have an effect on the free-living population of parasitic nematodes.

The most prevalent larvae identified in coprocultures were from the genus *Haemonchus*, followed by the genera *Cooperia* and *Oesophagostomum* (Table 1). The nematode genera prevalence data coincides with other research carried out in the same region (Oliveira *et al*. 2018; Vieira *et al*. 2020), as these genera are predominant in tropical climates (Heckler and Borges, 2016). These genera are the main responsible for the losses in ruminant breeding (Girão *et al*. 1999). The genus *Haemonchus* is a helminth that parasites the abomasum, where it feeds on blood, causing anemia and dehydration; when it occurs in large numbers, it can

result in host death (Girão *et al*. 1999). The higher prevalence of larvae of the genus *Haemonchus* is due to the fact that the females are more prolific than those of the genera *Cooperia* and *Oesophagostomum* (Furlong *et al*. 1985). *Cooperia* species cause damage to the digestive system that compromises the use of nutrients (Girão *et al*. 1999). *Oesophagostomum* alters the digestion and absorption of food, hindering the growth of animals (Girão *et al*. 1999). Due to these spoliative actions, the intense presence of endoparasites is linked to less weight gain or weight loss.

Although both groups of animals were parasitized by gastrointestinal helminths throughout the study period, the group treated with fungi had greater weight gain in the last month evaluated ($p \leq 0.05$). This can be explained by the better immunological ability of hosts exposed to low parasitic loads, which allows them to continue growing at a satisfactory rate (Amarante and Sales, 2007).

Life cycle stages of the main gastrointestinal parasites is direct and fast and it involves the animal and the pasture, so decrease in animals parasitism depends on the intervention in the helminths free-living stages population dynamics (Torres-Acosta and Hoste, 2008). The fungi combination *M. sinense* and *P. chlamydosporia* led to reductions in environmental contamination by gastrointestinal nematodes. The reductions in L3 recovered from pastures were 91.7% at a distance of 0–20 cm and 86.4% at 20–40 cm from the faecal pats. Faecal pats is an important reservoir of L3, even during prolonged periods of drought (Verschave *et al*. 2016) and it has suitable characteristics for fungal development. Peaks on gastrointestinal nematode larvae number in the control group were observed in the months of June, October, November and January. These L3 peaks may have been influenced by the greater number of eggs expelled in the months of May, November and December.

Climatic conditions affect the infective larvae number in pastures (Quadros *et al*. 2012). It was an important note that during the experimental period the height of the pasture varied from 30 to 60 cm, providing a favorable microclimate to development and larvae survival, besides it protects faecal pats from rapid desiccation and degradation (Rocha *et al*. 2008). Another factor that had a positive effect on L3 distribution was the average temperature recorded in the experimental period. According to Heckler and Borges (2016), temperature variations between 13°C and 26°C are adequate for maintaining the free-living stages of the genus *Haemonchus*, *Cooperia* and *Oesophagostomum*. The rain occurrence during the experimental time also contributed to the larvae incidence on pastures. The water presence is essential to provide L3 migration from faecal pats to pasture (Quadros *et al*. 2012; Van Dijk and Morgan, 2011). It is interesting to point that nematophagous fungi are more present in soil

with high organic matter and moisture levels (Zhang and Hyde, 2014), so the faecal pats is an excellent culture media for them. In addition, the microclimate of the pasture plus the bovine faecal pats protects the fungi from direct ultraviolet light incidence and other abiotic factors.

Brazilian pastures favor cattle breeding and comply by a great demand for food production. On the other hand, livestock farming management has affected harmful the environment. Therefore, bioproducts availability for controlling nematodiasis will immensely contribute to minimize negative impacts resulted by the disease and the excessive drugs use in animal production systems (Araújo *et al*. 2021; Mendoza-de-Gives *et al*. 2022). It must be considered that for bettering adherence by producers sustainable practices, a greater offer of biological products is necessary for use in livestock farming. In our study, we showed that nematophagous fungi combination *M. sinense* and *P. chlamydosporia* had a direct influence on infective larvae number in the pasture and on cattle parasitic load raised in pasture.

Author Contribution

Isabela and Jackson Victor conceived and designed the study. Isabela conducted data gathering. Ítalo performed statistical analyses. Isabela, Ítalo, Samuel, Artur and Jackson Victor wrote the article.

Ethical Considerations

This study was previously approved by the Animal Use Ethics Committee of the Federal University of Viçosa (protocol number 91/2018). The experimental test strictly followed all procedures recommended by the rules of conduct for the use of animals in teaching, research and extension of the Veterinary Department of the Federal University of Viçosa.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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8. CONCLUSÃO GERAL

Os testes de antagonismo em confrontação direta, antibiose e efeito de metabólitos voláteis entre os isolados SF53 e VC4 indicaram a viabilidade de crescimento em conjunto destes fungos. Além disso, a avaliação nematicida mostrou que a melhor eficácia foi quando os dois isolados foram usados em conjunto, com redução de 98,90% no número de L3 de nematoides de bovinos em condições *in vitro*.

A compreensão das variáveis ambientais é essencial para garantir o sucesso dos fungos nematófagos como agentes biocontroladores. A espécie *P. chlamydosporia* apresentou a maior taxa de crescimento em meio ágar água a 20°C, enquanto *M. sinense* apresentou um crescimento numericamente melhor a 30°C. Os fungos não cresceram a 35 ou 40°C durante os seis dias de observação. Surpreendentemente, o crescimento micelial de ambos os isolados inibidos na temperatura de 35°C por 6 dias, recomeçaram seu crescimento quando a temperatura foi reduzida para 25°C. Já a temperatura de 40°C foi prejudicial para o desenvolvimento de ambos os fungos. A observação do pH foi importante para mostrar que as variações de pH no trato gastrointestinal dos bovinos não serão prejudiciais aos fungos, uma vez que oferecer formulações orais aos animais é a forma mais prática de dispersar fungos nas fezes.

A microscopia eletrônica e óptica mostrou as estruturas fúngicas que beneficiam seu uso no controle biológico de nematoides e interações com larvas infectantes de helmintos. Larvas de nematoides mantidas pelo micélio de *P. chlamydosporia* confirmam sua capacidade de predar os estágios larvais, apesar de ser um fungo classificado no grupo "ovicida".

Na avaliação a campo, a recuperação de L3 da pastagem do grupo tratado com a combinação fúngica foi 91,7% e 86,4% menor do que na pastagem do grupo controle, respectivamente para amostras coletadas a 20 cm e 40 cm das fezes. Os valores de ovos por grama de fezes (OPG) nos meses de maio de 2019 e janeiro de 2020 foram menores ($p \leq 0.05$) no grupo tratado. Portanto, a utilização da combinação entre *M. sinense* e *P. chlamydosporia* nos sistemas de criação animal é uma ferramenta útil para reduzir a quantidade de larvas infectantes no meio ambiente.

ANEXO A

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 91/2018, intitulado "**Formulação associada entre fungos *Pochonia chlamydosporia* e *Monacrosporium sinense* no controle biológico de nematoides gastrintestinais de bovinos**", coordenado pelo professor Jackson Victor de Araújo do Departamento de Veterinária, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 21/02/2019, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 91/2018, named **"Associated formulation between fungi Pochonia chlamydosporia and Monacrosporium sinense in the biological control of bovine gastrointestinal nematodes"**, is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on February 21, 2019 valid for 12 months.

Prof.ª Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV