FILIPPE ELIAS DE FREITAS SOARES

PRODUÇÃO, PURIFICAÇÃO E IDENTIFICAÇÃO DE ENZIMAS EXTRACELULARES DE FUNGOS NEMATÓFAGOS E SUAS ATIVIDADES NEMATICIDAS

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

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"*Não há assuntos pouco interessantes; apenas há pessoas pouco interessadas*."

G. K. Chesterton

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BIOGRAFIA

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RESUMO

SOARES, Filippe Elias de Freitas, D.Sc., Universidade Federal de Viçosa, novembro de 2014. **Produção, purificação e identificação de enzimas extracelulares de fungos nematófagos e suas atividades nematicidas.** Orientador: José Humberto de Queiróz. Coorientadores: Fábio Ribeiro Braga e Jackson Victor de Araújo.

O controle biológico é uma das possíveis aplicações biotecnológicas de enzimas fúngicas. Este tipo de controle se destaca por ser uma alternativa "limpa", sem o uso de produtos químicos que possam gerar possíveis resíduos. Nesse contexto, fungos nematófagos produzem enzimas extracelulares envolvidas em diversas etapas da infecção, como liberação de nutrientes para o crescimento do microrganismo, penetração da cutícula pela degradação proteica e digestão do tecido hospedeiro. O mecanismo molecular da ação patogênica de fungos contra nematoides não é completamente conhecido. Dessa forma, o presente trabalho teve como objetivo otimizar a produção, purificar e caracterizar enzimas extracelulares produzidas por fungos nematófagos, bem como avaliar sua capacidade nematicida. Micélios obtidos da transferência de discos de cultura de três isolados de fungos nematófagos (*Monacrosporium thaumasium* (NF34), *M. sinense* (SF53) e *Pochonia chlamydosporia* (VC4)) mantidos em corn-meal-ágar 2% (CMA 2%) e transferidos para frascos contendo meio de cultura que teve sua composição otimizada para a produção de protease e quitinase. Em seguida, as enzimas foram purificadas e caracterizadas em relação ao pH e temperatura. Por fim, foi demonstrada a atividade nematicida dos extratos brutos e das enzimas purificadas sobre larvas de nematoides. Duas varíaveis (pH e tempo de incubação), apresentaram um efeito significativo ($p<0,05$) sobre a produção de protease do fungo nematófago *Monacrosporium sinense* (SF53). Além disso, este fungo produziu três diferentes proteases (Ms1, Ms2 e Ms3) com atividade nematicida sobre larvas de *Panagrellus redivivus*, sugerindo que essas proteases sejam produzidas como um complexo enzimático eficaz na destruição de larvas de primeiro estágio de *Angiostrongylus vasorum*. Em relação a *M. thaumasium* (NF34), foi observada atividade nematicida ($p<0.01$) de seus conídios e de seu extrato bruto proteolítico em coproculturas sobre larvas de primeiro estádio de *A. vasorum*. Os efeitos das variáveis (quitina, quitina coloidal, glicose, nitrato de sódio ($NaNO_3$), umidade) sobre a produção

de quitinase em fermentação no estado sólido pelos isolados de *Monacrosporium* NF34 e SF53 foram analisados usando o planejamento estatístico Plackett-Burman. Para o isolado NF34, nos níveis avaliados, a presença de quitina e $NaNO_3$ nos meios de cultura foram significativas ($p<0,05$) para a produção de quitinase. Por outro lado, para o isolado SF53, nos níveis avaliados, apenas a presença de glicose foi significativa ($p<0,05$) para a produção de quitinase. Em relação a influência do pH na atividade enzimática, para *M. thaumasium* (NF34), o valor de pH com maior atividade obtida foi 5.5. Entretanto, para *M. sinense* (SF53), a maior atividade foi observada em pH 8. Além disso, observou-se que o isolado NF34 produziu duas quitinases distintas com a atividade nematicida sobre larvas de *P. redivivus*. Por fim, a atividade ovicida do fungo nematófago *Pochonia chlamydosporia* (VC4) e de suas proteases e quitinases foi avaliada sobre ovos de *Dioctophyma renale*. Houve diferença significativa ($p <0,01$) na destruição de ovos em relação ao grupo de controle, não se verificando diferença estatisticamente significativa ($p> 0,01$) entre as concentrações de *P. chlamydosporia* na destruição de ovos. No entanto, houve uma tendência de aumento da mortalidade com o aumento da concentração de *P. chlamydosporia*. As proteases e quitinases do isolado VC4, causaram uma redução de percentagem significativa ($p <0,01$) no número de ovos viáveis de *D. renale*, em relação ao controle, com os seguintes valores de redução: 27,8% (proteases), 29,4% (quitinases) e 43,4% (proteases + quitinases). Mais estudos devem ser conduzidos no sentido de otimizar as condições de produção de enzima e aplicação destas no combate de nematoides.

ABSTRACT

SOARES, Filippe Elias de Freitas, D.Sc., Universidade Federal de Viçosa, November, 2014. **Production, purification and identification of extracellular enzymes from nematophagous fungi and their nematicidal activities.** Adviser: José Humberto de Queiróz. Co-adviser: Fábio Ribeiro Braga e Jackson Victor de Araújo.

Biological control is one of the possible biotechnological applications of fungal enzymes. This type of control is known for being a "clean" alternative, without the use of chemicals that may generate residues. In this context, nematophagous fungi produce extracellular enzymes that are involved in various stages of infection, such as release of nutrients for microorganism growth, cuticle penetration through protein degradation and digestion of host tissue. However, the detailed molecular mechanism of pathogenic action of fungi against nematodes is not complete known. Thus, the present study aimed to optimize production, purify and characterize extracellular enzymes produced by nematophagous fungi and evaluate their nematicidal ability. Mycelia were collected through the transfer of culture dishes of three isolates (*Monacrosporium thaumasium* (NF34), *M. sinense* (SF53) and *Pochonia chlamydosporia* (VC4)) of nematophagous fungi kept in 2% corn-meal-agar (2% CMA) and transferred to vials containing culture medium that had the composition optimized for the production of protease and chitinase. Then, the enzymes were purified and characterized with respect to pH and temperature. Finally, the nematicidal activity of crude extracts and purified enzymes on nematodes larvae was demonstrated. Two variables (pH and incubation time) showed a significant effect ($p < 0.05$) on the production of protease from nematophagous fungus *Monacrosporium sinense* (SF53). Moreover, this fungi produced three different proteases (Ms1, Ms2 and MS3) with nematicidal activity against *Panagrellus redivivus* larvae. It is also suggested that these proteases are produced in an enzymatic complex that was effective in the destruction of first-stage larvae of *Angiostrongylus vasorum*. Regarding *M. thaumasium* (NF34), it was observed nematicidal activity ($p < 0.01$) of its conidia and crude extract in proteolytic coprocultures on first stage larvae of *A. vasorum*. The effect of the variables (chitin, colloidal chitin, glucose, sodium nitrate ($NaNO_3$), moisture) on chitinase production in solid state fermentation by *Monacrosporium* isolates SF53 and NF34 were analyzed using Plackett-

Burman statistical design. For the isolate NF34, at the evaluated levels, the presence of chitin and $NaNO_3$ in culture medium was significant ($p < 0.05$) for the production of chitinase. On the other hand, for isolate SF53, at the evaluated levels, only the presence of glucose was significant ($p < 0.05$) for chitinase production. Regarding the influence of pH on enzyme activity, for *M. thaumasium* (NF34) the pH value with the highest activity was 5.5. However, for *M. sinense* (SF53), the highest activity was observed at pH 8. Furthermore, it was observed that the isolate NF34 produced two different chitinases with nematicidal activity against larvae of *P. redivivus*. Finally, the ovicidal activity of the nematophagous fungus *Pochonia chlamydosporia* (VC4) and its proteases and chitinases was evaluated on *Dioctophyma renale* eggs. There was a significant difference ($p < 0.01$) in the destruction of eggs in relation to the control group. Moreover, there was no difference ($p > 0.01$) between the concentrations of *P. chlamydosporia* in the destruction of eggs. However, there was a trend for increased mortality with increasing its concentration. Proteases and chitinases of isolate VC4 have caused a significant percentage reduction ($p < 0.01$) in the number of viable eggs of *D. renale*, compared to control, with the following values of reduction: 27.8% (proteases), 29 4% (chitinase) and 43.4% (proteases + chitinases). More studies should be conducted to optimize enzyme production conditions and the application of these on nematodes.

1. INTRODUÇÃO GERAL

O controle biológico é uma das possíveis aplicações biotecnológicas de enzimas fúngicas, evitando o uso de produtos químicos que possam gerar possíveis resíduos. Nesse contexto, o controle biológico realizado com fungos nematófagos é uma alternativa que apresenta grande potencial. Os fungos nematófagos são inimigos naturais de nematoides que podem capturar, matar e digerir os ovos e larvas infectantes de nematoides utilizando hifas especialmente modificadas chamadas armadilhas. Esses fungos produzem enzimas extracelulares envolvidas em diversas etapas da infecção, como liberação de nutrientes para o crescimento do microrganismo, penetração da cutícula pela degradação proteica e digestão do tecido hospedeiro (Mendoza de Gives et al. 2003; Yang et al. 2013; Braga e Araújo, 2014).

Os nematoides do gênero *Panagrellus* têm distribuição cosmopolita e são utilizados como modelo de experimentação envolvendo sua interação com fungos nematófagos (Gomes et al. 1999; Braga et al 2012a). Outro nematoide de interesse é o *Angiostrongylus vasorum* (Baillet, 1866) Kamensky, 1905, um protostrongilídeo parasito de cães domésticos e canídeos silvestres. O parasito adulto pode ser encontrado no ventrículo direito, artérias pulmonares e suas ramificações, com conseqüências graves para o hospedeiro definitivo. As larvas desse nematoide podem se encontrar livres no ambiente, levando a possibilidade de infecção humana, uma vez que outros parasitos do gênero *Angiostrongylus* são comprovadamente zoonóticos (Barçante et al., 2003; Oliveira-Jr et al., 2006). Pode-se destacar ainda o verme renal gigante, *Dioctophyma renale*, o maior nematoide conhecido. (Bowman et al., 2006). Em seres humanos, a infecção ocorre devido ao hábito de consumir peixe, carne de rã cru, ou anelídeos aquáticos com as larvas infectantes (L_3). Nesse sentido, medidas alternativas que possam ser empregadas no combate à disseminação ambiental, destes parasitos e suas formas infectantes são importantes, como o uso de fungos nematófagos.

Recentemente vários trabalhos que envolvem a produção e a ação nematicida das proteases produzidas por fungos nematófagos foram realizados. Soares et al. (2012) demonstraram que uma serino protease produzida pelo fungo nematófago *Monacrosporium thaumasium* (NF34) apresentou atividade larvicida sobre larvas de primeiro estádio de *A. vasorum*.

Braga et al. (2012b) purificaram uma serino protease produzida pelo fungo nematófago *Duddingtonia flagrans* (AC001) com atividade larvicida sobre larvas infectantes de ciatostomíneos, nematoide parasito gastrintestinal de equinos. Anteriomente, Lopez-Llorca (1990) demonstrou que o fungo nematófago *Verticillium suchlasporium* produz a protease P32 com ação sobre ovos de fitonematoides. Segers et al. (1994) mostraram que o mesmo fungo secretou outra protease (VCP1) que hidrolisou as proteínas externas da casca do ovo do nematoide parasita de planta *Meloidogyne incognita*, expondo a camada de quitina. Khan et al. (2004) demonstraram que serino proteases secretadas por *Paecilomyces lilacinus* foram capazes de interromper o desenvolvimento de ovos de *M. javanica* e reduzir o número de juveniles. Proteases e quitinases purificadas também demonstraram atividade nematicida sobre *Haemonchus contortus,* parasita gastrintestinal de ruminantes domésticos *(*Mansfield et al., 1992). Braga et al. (2010) demonstraram que o extrato bruto enzimático de *Pochonia chlamydosporia*, fungo nematófago do grupo ovicida, apresentou atividade efetiva sobre larvas e ovos de nematoides.

Os fungos nematófagos podem ser classificados em quatro grupos: endoparasitas, predadores, ovicidas ou oportunistas, e produtores de metabólitos tóxicos, que produzem metabólitos secundários nematicidas (Yang et al. 2013). *Pochonia chlamydosporia* produz metabólitos do tipo aurovertina, com atividade nematotóxica sobre o fitonematoide *Meloidogyne incognita.* Os metabólitos secundários produzidos por fungos nematófagos também possuem um grande potencial para serem utilizados como controladores biológicos (Niu et al. 2010).

A casca de ovo de nematoides contém fibrilas de quitina embebidas numa matriz de proteína, sendo o complexo de quitina provavelmente a principal barreira contra a infecção fúngica (Wharton 1980; Bird e Self, 1995; Yang et al., 2013). Recentemente, foi identificada a primeira quitinase (CHI43) de dois fungos nematófagos *Verticillium chlamydosporium* (syn. *Pochonia chlamydosporia*) e *Verticillium suchlasporium* (syn. *Pochonia rubescens*) com atividade nematicida (Tikhonov et al., 2002). Além disso, Khan et al. (2003) demonstraram que *Paecilomyces lilacinus* produz uma quitinase que danificou drasticamente as estruturas da casca de ovos de *Meloidogyne javanica* (Khan et al. 2004). No entanto, pouco ainda se sabe sobre outras quitinases de

fungos nematófagos. A ação patogênica desses fungos contra nematoides ainda precisa de maiores estudos para a sua elucidação (Braga e Araújo, 2014; Yang et al., 2013).

Além disso, para que o controle biológico com fungos nematófagos seja incorporado em um sistema industrial de produção, o mesmo deve ser estudado em relação a sua atividade predatória, capacidade de produzir enzimas extracelulares de importância no processo de infecção, bem como a ação destas enzimas sobre nematoides dos mais diversos gêneros (Araújo et al., 2004; Braga et al., 2010a,b; Soares et al., 2012).

O desenvolvimento de alternativas ao uso de anti-helmínticos são importantes. Nesse contexto, destacam-se as enzimas com atividade nematicida produzidas por fungos nematófagos (Araújo et al., 2008; Braga et al., 2009), as quais ainda carecem de maiores estudos em relação à produção, purificação e interação com os nematoides, o que poderá contribuir no controle integrado destes patógenos.

2. OBJETIVOS

2.1. Objetivo Geral

Otimizar a produção, purificar e testar a ação nematicida das enzimas extracelulares dos fungos nematófagos *Monacrosporium thaumasium* (NF34), *M. sinense* (SF53) e *Pochonia chlamydosporia* (VC4).

2.2. Objetivos Específicos

Otimizar as condições de cultura dos fungos nematófagos *Monacrosporium thaumasium* (NF34) e *M. sinense* (SF53) para uma elevada produção de enzimas utilizando o planejamento fatorial Plackett-Burman e metodologia de superfície de resposta;

Purificar as enzimas produzidas por *Monacrosporium thaumasium* (NF34), *M. sinense* (SF53);

Avaliar a atividade nematicida dos extratos brutos e das enzimas purificadas dos fungos nematófagos *Monacrosporium thaumasium* (NF34), *M. sinense* (SF53) e *Pochonia chlamydosporia* (VC4) sobre larvas de *Panagrellus redivivus* e *Angiostrongylus vasorum* e ovos de *Dioctophyma renale.*

CAPÍTULO 1

Nematicidal activity of three novel extracellular proteases of the nematophagous fungus *Monacrosporium sinense*

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Nematicidal activity of three novel extracellular proteases of the nematophagous fungus *Monacrosporium sinense*

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Abstract

Extracellular proteases are an important virulence factor for the nematophagous fungi *Monacrosporium*. The objective of this study was to optimize, purify, partially characterize and to evaluate the nematicidal activity of the proteases produced by the nematophagous fungus *Monacrosporium sinense* (SF53) by solid state fermentation. Wheat bran was used as substrate for protease production. The variables moisture, pH, incubation time, temperature, glucose, yeast extract and the number of conidia were tested for their influences on protease production by SF53. To determine the optimal level of the selected variables the Central Composite Design (CCD) was applied. The crude extract obtained was purified in two steps, an ion exchange chromatography and a gel excision. SDS-PAGE and zymogram were performed for analysis of the purification process. Proteolytic activity was also tested at different pHs and temperatures. In the *in vitro* assay, the nematicidal activity of the three proteases was evaluated. pH and incubation time showed a significant effect ($p < 0.05$) on production of protease. The highest value of activity was 38.0 (U/ml) under the conditions of pH 5.0 and incubation time of 211 hours. SF53 produced three different proteases (Ms1, Ms2 and Ms3) which were directly purified from the zymogram. Ms1, Ms2 and Ms3 showed the following percentage of reduction ($p < 0.05$) on the number of *P. redivivus* compared to control after 24 hours: 76.8%; 68.1% and 92.1%. This is the first report of the use of proteases of the isolate SF53 on a phytonematode, which may be a research tool in future works.

Keywords: Nematophagous fungi; *Monacrosporium sinense; Panagrellus redivivus;* proteases; Plackett-Burman; Central Composite Design

Introduction

Stock and Nadler (2006) have report that the phytonematode *Panagrellus* has global distribution. Their species have been described in almost all continents, with the exception of Antarctica and Australia. The species *P. redivivus* described by Linnaeus (1767), has been used as an experimental model for nematicidal assays with nematophagous fungi, due to their ease of maintenance and use in biological control assays. In this sense, Cardoso et al. (2009) demonstrated the activity of nematophagous fungi against this phytonematode, registering potential effectiveness. However, there are no reports of the enzymatic activity of nematophagous fungi on *P. redivivus*.

Nematophagous fungi can be exploited for industrial production due to their ability to grow on solid substrates and produce a wide range of extracellular enzymes (Araújo et al. 2000; Sharma et al. 2007). In previous works Soares et al. (2012a, b) showed that nematophagous fungi from the *Monacrosporium* genus produce proteases with possible nematicidal activity, which may be exploited in the future as biocontroller. However, many factors such as sources of carbon and nitrogen, the quantity of inoculum; initial pH, temperature, moisture and time of fermentation have been reported as influent parameters in the fermentation process and therefore in the production of proteases.

On the other hand, the literature mentions that the main source for enzymes production are the microorganisms, which currently have been most studied, since they are able to possible biotechnological applications (Rao et al. 1998; Tunga et al. 2003**).** Further, according to Rai and Mujerj (2009), the enzymes are considered chemically-bio-supporting products, with a great variety of industrial and biotechnological applications.

Thus, the objective of this study was to optimize, purify, partially characterize and to evaluate the nematicidal activity of the proteases produced by the nematophagous fungus *Monacrosporium sinense* (SF53) by solid state fermentation.

Materials and methods

Fungi

An isolate of the nematophagous fungus *M. sinense* (SF53) originating from soil of Brazil, in the city of Viçosa, Zona da Mata of Minas Gerais, was used. This isolate has been kept in test tubes at 4 °C containing 2% corn-meal-agar (2% CMA) by continuous transfer in the dark for 10 days.

Panagrellus redivivus

The free-living nematode *P. redivivus* is originated from the Laboratory of Parasitology, Department of Veterinary Medicine, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil. For its maintenance, 1.0 ml of an aqueous suspension with approximately 1000 *P. redivivus* was inoculated in each sterile polystyrene petri dish containing thin oat flakes medium and water in a 1:1 ratio, according to Araujo et al. (2004).

Production of conidia and culture conditions

Conidia produced by *M. sinense* (SF53) were placed in sterile saline to prepare the inoculum for fermentation. Conidia were counted using a Neubauer chamber with light microscopic, 10x objective and serially diluted to obtain the desired concentration (10^4 or 10^5 conidia/mL). The liquid medium used for the production of enzyme was established in grams per liter: K_2HPO_4 (5.0 g/l), $MgSO_4$ (0.10 g/l), $ZnSO_4$ (0.0050 g/l);

$FeSO_4$ (0.001 g/l) and $CuSO_4$ (0.50 mg/l), according to modified methodology of Braga et al. (2011).

Solid-state fermentation (SSF)

Wheat bran was used as substrate for protease production. Fermentations were conducted in Erlenmeyer flasks (125 ml) containing 5 g of wheat bran. The moisture was adjusted using a liquid medium, by varying the volume/weight ratio according to the following experimental design. Each flask was covered with hydrophobic cotton and autoclaved at 121 °C for 15 min. After cooling, each flask was inoculated with conidia suspension previously prepared and incubated at 28 °C in BOD for different time periods defined by the experimental design.

Statistical design

Plackett-Burman experimental design (Plackett and Burman 1946) is used to analyze and evaluate the important factors that affect a polynomial first-order response. In the present work, this statistical design was used to scan some variables inherent to the fermentation regarding their influences on protease production by the fungus *M. sinense* (SF53). The variables moisture, pH, incubation time, temperature, glucose, yeast extract and the number of conidia were examined at two levels: -1 to the low level and +1 to the high level (Table 1).

Table 1. High (+1) and low (-1) levels of the seven analyzed variables (moisture (%), pH, incubation time, temperature, glucose (g/l), yeast extract (g/l) and number of conidia) in the Plackett-Burman statistical design

Variables	High level (+1)	Low level (-1)
Moisture (%)	200	100
pH	5	3
Incubation time (hours)	5	3
Temperature	28	35
Glucose (g/l)	1	0
Yeast extract (g/l)	1	0
Number of conidia	10^5	10^4

This methodology was based on previous work of Soares et al. (2012b) about the protease production of fungus from *Monacrosporium* genus. Then, Minitab 15 software was used for analyze. The number of experiments was n + 1 according to experimental design Placket-Burmann, where n is the number of variables. The experimental design in which each line represents one experiment and each column represents an independent variable can be seen in Table 2. The proteolytic activity was measured in triplicate, and the mean of these values was used as the response Y.

Table 2. Matrix of the Plackett-Burman experimental design of protease production (U/ml) by the nematophagous fungus *Monacrosporium sinense* (SF53)

Run	Moisture (%)	pH	Incubation time (hours)	Temperature	Glucose (g/l)	Yeast extract (g/l)	Number of conidia	Acivity (U/ml)
1	200	3	5	28	0	0	5	13.5
2	200	5	3	35	0	0	4	7.85
3	100	5	5	28	1	0	4	15.48
4	200	3	5	35	0	1	4	5.45
5	200	5	3	35	1	0	5	6.75
6	200	5	5	28	1	1	4	24.1
7	100	5	5	35	0	1	5	19.2
8	100	3	5	35	1	0	5	5.45
9	100	3	3	35	1	1	4	3.85
10	200	3	3	28	1	1	5	6.2
11	100	5	3	28	0	1	5	8.2
12	100	3	3	28	0	0	4	0

Response surface methodology

To determine the optimal level of the selected variables from the statistical design Plackett-Burman (pH and incubation time) and investigate their interactions, the response surface methodology was applied using the Central Composite Design (CCD). The effect of these significant variables on enzyme activity was studied in 5 experimental levels: -α, -1, 0, 1, + α, where $\alpha=2^{n/4}$, n is equal to the number of variables and 0 corresponds to the central point.

A total of 13 experiments, with five replicates at the central point were performed. All analyzes were performed in triplicate. The Design Expert 7.0 software was used to analyze experimental data. The CCD is presented in Table 3.

Table 3. Analysis of the studied variables (moisture (%), pH, incubation time, temperature, glucose (g/l), yeast extract (g/l) and number of conidia) in the Plackett-Burman statistical design

	Effect	**Coef**	**SE Coef**	**t-*test***	**P>|*t*|**
Constant		14.7536	2.1048	7.01	0.002
Moisture (%)	1.945	0.9725	1.1796	0.82	0.456
pH	7.855	3.9275	1.1796	3.33	0.029
Incubation time (hours)	16.7767	8.3883	2.3592	3.56	0.024
Temperature	-1.8029	-0.9014	0.6741	-1.34	0.252
Glucose (g/l)	1.2717	0.6358	1.1796	0.54	0.618
Yeast extract (g/l)	2.995	1.4975	1.1796	1.27	0.273
Number of conidia	0.8567	0.4283	2.3592	0.18	0.865

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

The relationship between the variables and the response (enzymatic activity) was calculated using the following polynomial equation:

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

Where Y is the response variable, a_0 is the coefficient of interception, a_i is the coefficient of the linear effect, a_{ii} is the coefficient of the quadratic effect and a_{ij} is the coefficient of interaction effect. x_i and x_j denote the levels of the coded variables X_i and X_j in the experiments.

Enzyme extraction

The extraction of the protease produced by SSF was performed according to Soares et al. (2010), with modifications. To the flasks containing the fermented material, distilled water (5:1, v/w) was added by stirring in a rotary shaker at 200 rpm

for 30 min at room temperature. The suspension was filtered through cheesecloth. Then all content obtained was centrifuged at 10,000 xg for 10 min at 4 °C to remove insoluble materials. The clear supernatant was used for the assays.

Purification of the proteases

The first purification step was an ion-exchange chromatography. The clear supernatant obtained from the enzyme extraction was applied to a DEAE-Sepharose column previously equilibrated in Tris-HCl 50 mM pH 8.0, with a flow adjusted to 1.0 ml per minute. Proteins bound to the column were eluted using the same buffer with NaCl 0.5 M. Fractions with protease activity were collected in a pool. These fractions were separated on a 10% polyacrylamide gel with co-polymerized casein (casein-SDS-PAGE) according to the methodologies of Braga et al. (2012a) and Nagarathnam et al. (2010). The samples were mixed with running buffer (without β-mercaptoethanol) and applied without preheating. After electrophoresis, the gel was incubated with a solution of 2.5% Triton X-100 for 1 hour, followed by three washes with distilled water. Then the gel was incubated in Tris-HCl 50 mM (pH 8.0) for 30 min at 50 °C. For revelation, the gel was stained with Coomassie Brilliant Blue R-250. The action of the enzymes can be observed by the formation of white halos. The protein bands were then excised from the gel and ground in a mortar using 50 mM Tris-HCl (pH 8.0) and centrifuged at 8000 × g for 15 min at 4 °C to remove the gel particles. The supernatant was used for the characterization assays. Elution was monitored by enzymatic assay and measurement of protein content described by Bradford (1976). A standard curve was constructed for quantification of the protein content, using bovine serum albumin. The entire purification procedure was conducted at 4 °C. The purified proteins were used in subsequent analyzes.

Partial characterization of proteases

The optimum pH for activity of the proteases was determined by incubating the reaction mixture in pH values of 5.0 to 10.0 at 50 °C. The optimum temperature for the activity of the proteases was determined by incubating the reaction mixture at temperatures of 30-70 °C, using the pH of maximum activity obtained in the above assay.

Enzymatic assay

The activity of the proteases of *M. sinense* (SF53) was measured by the caseinolytic method, according to Soares et al. (2012a). A standard curve of tyrosine was built for the quantification of enzymatic activity. One unit of protease was defined as the amount of enzyme required to liberate 1.0 µg of tyrosine per minute under the assay conditions.

Electrophoretic analysis

Electrophoresis (SDS-PAGE) was performed to proteic analysis, as described by Laemmli (1970) using 10% polyacrylamide gel. The gel was silver stained to allow visualization of proteins. Furthermore, a zymogram was performed with casein as substrate (casein-SDS-PAGE) according Braga et al. (2012a). The enzyme action can be observed by the formation of white halos.

Nematicidal assay

The *in vitro* nematicidal assay was based on previous work of Soares et al. (2012b). Two groups were formed in sterile tubes for each enzyme, a treated group containing the purified enzyme and a control group (no enzyme). The tubes were

incubated at 28 °C in the dark for 24 hours. Fifty *P. redivivus* were poured in the sterile tubes. Control group contained only 50 larvae in distilled water. Three repetitions were performed for each group. After 24 hours, the total number of larvae tube present in each of the treated groups and control was counted with the aid of light microscopy. The data obtained in this assay were interpreted by analysis of variance significance levels of 1 and 5% probability. The efficiency of predation on larvae in relation to the control was measured by the Tukey test at 1% probability (Ayres et al., 2003). Subsequently, the mean percent reduction of larvae was calculated according to the following equation:

$$\% = \frac{(\bar{x}\text{larvae recovered from control} - \bar{x}\text{ larvae recovered from treatment})}{\bar{x}\text{larvae recovered from control}} \times 100$$

3.0. Results

Statistical design

The effects of seven variables (moisture, pH, incubation time, temperature, glucose, yeast extract and number of conidia) on protease production of *M. sinense* (SF53) by solid state fermentation were analyzed using the statistical design Plackett-Burman (Tables 1 and 2). By analysis of the studied factors, it was found that two of the culture parameters (pH and incubation time), in the assessed levels, showed a significant effect ($p < 0.05$) on protease production (Table 3). The R^2 of the model was 87.55% demonstrating the reliability of the model.

Response surface methodology

From the results of the statistical design Plackett-Burman, one of the response surface methodologies, the central composite design, was applied to determine the optimal levels of these two variables (pH and incubation time). For this, a total of 13

assays with different combinations of selected factors were performed. The central point was repeated five times to estimate the error. The results for the full factorial experimental design are shown in Table 4.

Table 4. Experimental design with 13 runs used for the realization of response surface methodology using two variables (pH and incubation time) each with 5 levels, and their respective values of proteolytic activity

Run Order	pH	Incubation time (hours)	Proteolytic activity (U/ml)
1	4.0	96	8.76
2	6.0	96	17.8
3	4.0	192	19.4
4	6.0	192	24.8
5	3.58	144	5.11
6	6.41	144	13.5
7	5.0	76.1	4.02
8	5.0	211	38.0
9	5.0	144	26.3
10	5.0	144	24.4
11	5.0	144	31.1
12	5.0	144	30.7
13	5.0	144	21.9

The final answer which provides proteolytic activity after the removal of terms relating to non-significant variables ($p > 0.05$) can be obtained by the function:

$$\mathbf{Y = - 251.94377 + 0.55125\ X_2 - 8.14500\ X_1^2}$$

Where Y is the response value (enzymatic activity) and X_1 and X_2 are coded levels of pH and incubation time, respectively.

The regression model had its statistical significance tested by the F test, and the variance analysis (ANOVA) was used for the response surface quadratic model (Table 5). The R^2 of the model was 0.85 demonstrating the reliability of the quadratic model. In the Figure 1, the three-dimensional response surface generated in accordance with the final model of protease production by the fungus *M. sinense* (SF53) can be observed.

Table 5. Analysis of variance for the response equation developed in the protease production by the nematophagous fungus *Monacrosporium sinense* (SF53) in solid-state-fermentation

Source	**SS**	**DF**	**MS**	***F*-value**	***P* > *F***
pH					0.1181
Incubation time					0.0030
pH*Incubation time					0.7442
pH^2					0.0046
Incubation $time^2$					0.2853
Model	1101.967	5	220.3933	8.016033	0.0082
Lack-of-Fit	129.1904	3	43.06348	2.722607	0.1788
Error	63.268	4	15.817		
Total	1294.425	12			

SS: sum of squares; DF: degrees of freedom; MS: mean of squares.

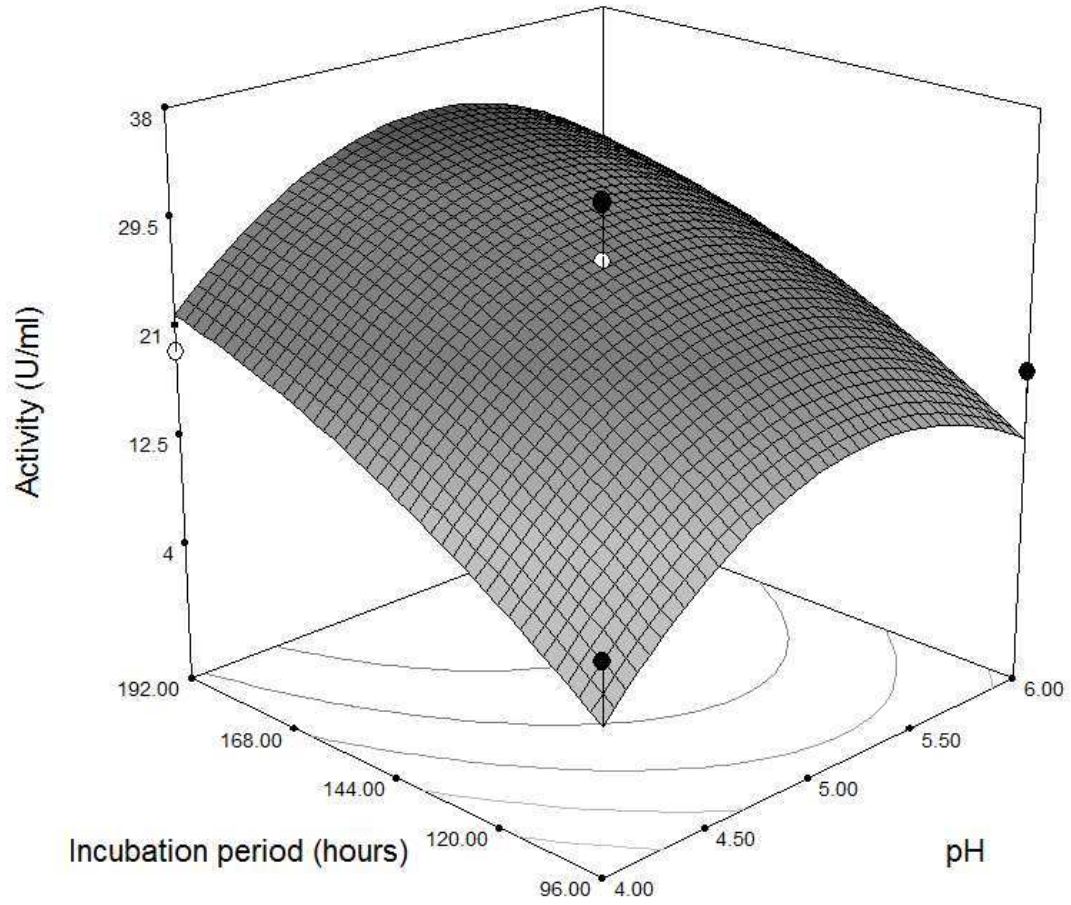


Fig. 1. Response surface curve of protease production by the nematophagous fungus *Monacrosporium sinense* (SF53) in solid state fermentation

Purification of the proteases

The extraction of protease produced by SSF generated a crude enzymatic extract with high protein level. On the other hand, after the purification process and subsequent analysis of the zymogram and SDS-PAGE, one can observe that the isolate SF53 produced three different proteases (Figure 2a,b). Purification factors and yields of each step of purification are presented in Table 6. The molecular weights of the purified enzymes (Ms1, Ms2 and Ms3) were approximately: 37 kDa, 33kDa and 11kDa (Fig. 2a).

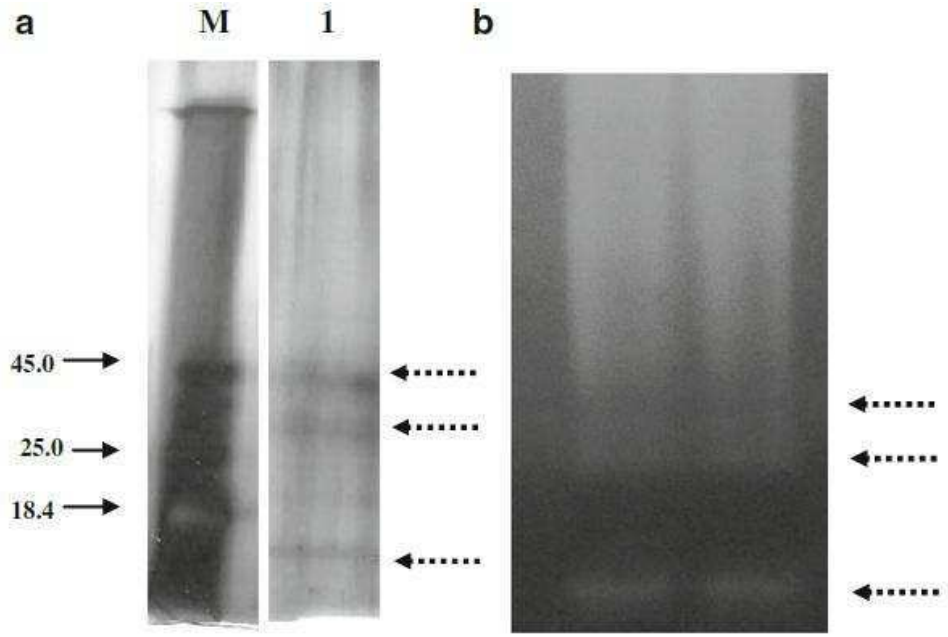


Fig. 2a- Purification analysis of the proteases produced by *Monacrosporium sinense* (SF53) in solid-state-fermentation through SDS-PAGE 10% gel **b**. Zymogram of the crude enzymatic extract produced by *M. sinense* (SF53) in solid state fermentation which suggests the production of at least three different proteases (Figure 2a,b). Line M: molecular weight marker (kDa); Line 1: Purified proteases. Dashed arrows: a. Proteases produced by *Monacrosporium sinense* (SF53). **b**. Action of the enzymes observed by the formation of white halos.

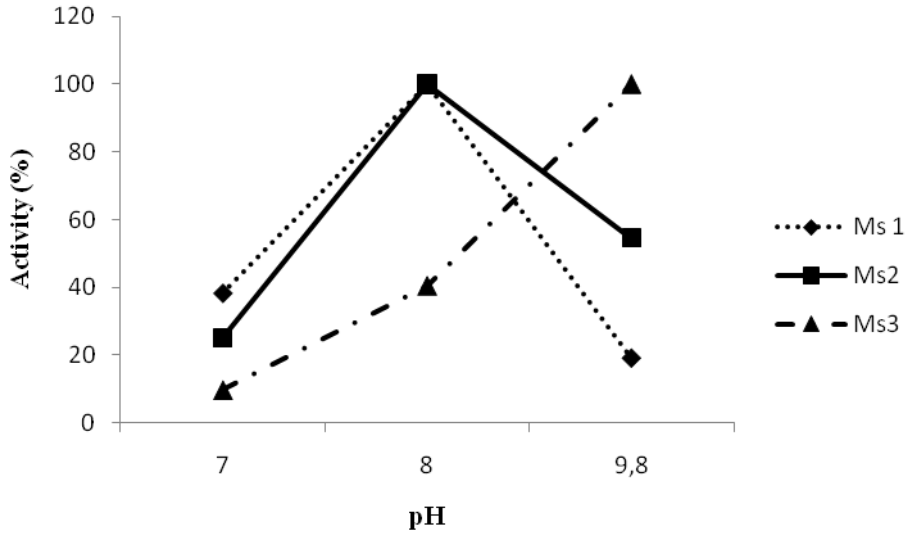
Table 6. Purification procedure of the proteases (Ms1, Ms2 and Ms 3) of *Monacrosporium sinense* (SF53)

Purification steps	Activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	261	718	0.36	100	1
DEAE-Sepharose	69	156	0.44	26.4	1.22
Gel (Ms1)	24.8	23.4	1.06	9.5	2.40
Gel (Ms2)	16.1	12.5	1.29	6.16	2.93
Gel (Ms3)	34.3	27.8	1.23	13.1	2.80

Partial characterization of proteases

The profile of proteolytic activity of the enzymes Ms1, Ms2 and Ms3 at different pHs and temperatures can be seen in Figure 3a, b. The highest activity of proteases Ms1 and Ms2 was obtained at pH 8.0. However, for Ms3, the highest activity was observed at pH 9.8 (Fig. 3a). In relation to the activity of the proteases at different temperatures, the highest value was observed at 60 ° C for all the enzymes (Fig. 3b).

a



b

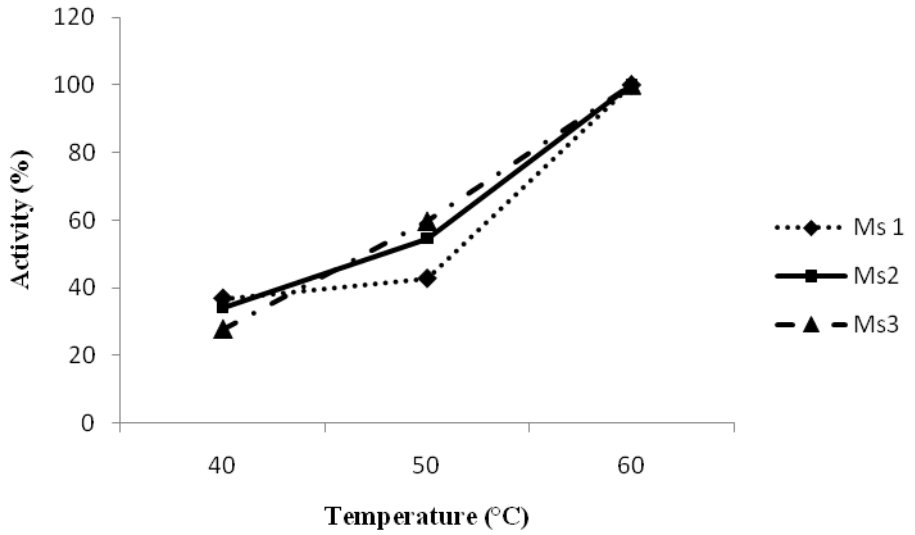


Fig. 3a. Effect of pH on relative activity (%) of proteases (Ms1, Ms2 and Ms3) of *Monacrosporium sinense* (SF53), in Tris-HCl 50 mM buffer (pHs 7.0, 8.0 and 9.8). The assay temperature was 60 °C; **b.** Effect of temperature on relative activity (%) of proteases (Ms1, Ms2 and Ms3) of *M. sinense* (SF53). Different values of temperature (40 ºC, 50 ºC and 60 ºC) were used at the pH of maximum activity obtained in the previous assay.

Nematicidal assay

The proteases (Ms1, Ms2 and Ms3) of *M. sinense* (SF53) showed the following percentage of reduction ($p < 0.05$) on the number of *P. redivivus* compared to control after 24 hours: 76.8%; 68.1% and 92.1%.

4. Discussion

The growth of nematophagous fungi is related to the carbon: nitrogen (C: N) and their predatory activity is stimulated by the presence of nematodes or substances derived, and the nematode acts as a major source of nitrogen (Araújo et al. 2004; Nguyen et al. 2007; Yang et al. 2011). In this context, the authors of the present work evaluated the influence of C and N sources on protease production by the isolate SF53 of the *Monacrosporium* genus.

Plackett-Burman statistical design was used to investigate the significance of seven variables on protease production by SF53. The results of the statistical analysis are shown in Table 3. The following tested variables showed no significant effect ($p > 0.05$) on the production of protease: Moisture ($P>|t|$) 0.456; Temperature ($P>|t|$) 0.252; Glucose ($P>|t|$) 0.618; Yeast extract ($P>|t|$) 0.273; Number of conidia ($P>|t|$) 0.865. However, only the variables pH ($P>|t|$) 0.029 and incubation time ($P>|t|$) 0.024 showed significant effect on the production of protease. In the present work, the isolate SF53 produced enzyme by solid state fermentation, since SF53 has used wheat bran as nitrogen source. However, when yeast extract was used as a supplementary source, the same was not significant ($p > 0.05$) on the production of proteases. In another study, Braga et al. (2011) reported that the nitrogen source, in this case casein, was significant for the production of protease by the nematophagous fungus *D. flagrans* (AC001).

The central composite design was applied to the determination of the optimal levels of the selected variables as significant by the statistical design (pH and incubation time). In the present study, the quadratic model was significant with a value of P>F 0.0082. Moreover, the lack of fit of the model was not significant as observed by the value of P>F 0.1788. The highest activity observed was of 38.0 (U/ml) under the conditions of pH 5.0 and incubation time of 211 hours. These results are in agreement with reports of Braga et al. (2011) about the proteolytic activity of the fungus *D. flagrans* (AC001).

The results indicated that the linear term of incubation time and the quadratic effect of pH had a significant effect ($p < 0.01$) on protease production by SF53. However, the linear effect of pH, the quadratic effect of incubation time and the interaction effect between pH and incubation time, did not demonstrate significance. In relation to these effects, there is a lack of studies in the literature aiming the optimization of enzyme production by nematophagous fungi. Thus, in recent work Soares et al. (2010) reported that the nematophagous fungus *P. marquandii* had its protease production by solid state fermentation optimized using central composite designIn that study, the authors also reported that the linear effect of incubation time and the quadratic effect of pH were significant for the process.

Regarding the response surface, figure 1, it was observed that the increase in incubation time and the decrease of pH caused an increase in the studied response (protease production by the isolate SF53). Moreover, some studies have reported that the pH can directly influence enzyme production by nematophagous fungi (Meyer and Wiebe, 2003; Esteves et al., 2009; Yang et al., 2011).

Biological control has been presented as one of the possible biotechnological applications. In this context, some researchers suggest that proteases are directly

involved in the larvicidal action on nematodes (Khan et al., 2003; Braga et al., 2011; 2012). Further according to Wang et al. (2006), Yang et al. (2008) and Soares et al. (2012a) the *Monacrosporium* genus produces proteases that play an important role during the infection process of nematode. Similarly in the present study it was observed that the isolate SF53 of *Monacrosporium* genus produced three proteases (Ms1, Ms2 and MS3), which is an interesting fact and that can be confirmed by zymogram and SDS-PAGE analysis. (Fig. 2a,b). Furthermore, the temperature at which the highest activity value was observed for the proteases (Ms1, Ms2 and Ms3) (60 °C) was identical to the optimum temperatures of serine proteases produced by other isolates of the *Monacrosporium* genus (Soares et al., 2012a; Wang et al., 2006; Yang et al., 2008). However, we can observe that the protease Ms3 has remarkable features such as low molecular weight and optimum activity at a very alkaline pH.

Regarding the nematicidal activity, was observed that the three enzymes produced by isolate SF53 demonstrated to be effective in the infection process of nematodes. The evidence of this activity could be observed through the obtained reduction percentages. In this context, as mentioned above, nematophagous fungi can be important biocontrollers of phytonematodes (Cardoso et al., 2009). However, it should be mentioned here that most of the studies use grown fungi, germinative structures (conidia and or chlamydospores) and mycelial mass (Braga et al., 2009; 2012b). However, this is the first report of the use of proteases of the isolate SF53 on a phytonematode, which may be a research tool in future works.

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CAPÍTULO 2

The nematophagous fungus *Monacrosporium thaumasium* and its nematicidal activity on *Angiostrongylus vasorum*

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The nematophagous fungus *Monacrosporium thaumasium* and its nematicidal activity on *Angiostrongylus vasorum*

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Abstract

Background: The dog acts as a reservoir and environmental disseminator of potentially zoonotic parasites. *Aims:* The objective of this work was to study the fungus *Monacrosporium thaumasium* regarding its nematicidal potential in laboratory trials and its proteolytic profile. *Methods:* The *in vitro* test was carried out through two assays (A and B). In assay A, conidia of the fungus N34a were added in positive coprocultures for *A. vasorum*. In assay B, crude extract (treated group) and distilled water (control group) were added to coprocultures. Next, the proteolytic profile of crude extract of the nematophagous fungus *M. thaumasium* (NF34a) was revealed by performing a zymogram. *Results:* There was a reduction ($p<0.01$) between the averages of larvae recovered from the treated groups (conidia and crude extract) in relation to control groups. The zymogram suggested that the nematophagous fungus *M. thaumasium*

produces a protease of approximately 40 kDa. *Conclusions:* The results of this work confirm that the conidia as well as the crude extract of the fungus *M. thaumasium* may be used to control *A. vasorum* L_1. The proteolytic profile suggested the presence of one protease (Mt1) of approximately 40 kDa that in the future may be used in biological control of L_1 of this nematode.

Keywords: Nematophagous fungi; *Angiostrongylus vasorum*; *Monacrosporium thaumasium*; protease.

El hongo nematófaga *Monacrosporium thaumasium* y su actividad nematicida sobre *Angiostrongylus vasorum*

Resumen

Antecedentes: El perro actúa como un reservorio y diseminador ambiental de parásitos potencialmente zoonóticos. *Objetivos*: El objetivo de este trabajo fue estudiar el hongo *Monacrosporium thaumasium* con respecto a su potencial nematicida en pruebas de laboratorio y su perfil proteolítico. *Métodos:* El ensayo in vitro fue realizado mediante dos ensayos (A y B). En el ensayo A, conidias del hongo N34A se añadieron en coprocultivos positivos para *A. vasorum*. En el ensayo B, extracto bruto (grupo tratado) y agua destilada (grupo de control) se añadieron a coprocultivos. A continuación, el perfil proteolítico de extracto crudo del hongo nematófaga *M. thaumasium* (NF34a) se reveló mediante la realización de un zimograma. *Resultados:* Hubo una reducción ($p < 0,01$) entre las medias de larvas recuperadas de los grupos tratados (conidios y el extracto bruto) en relación con los grupos de control. El zimograma sugirió que el nematófago hongo *M. thaumasium* produce una proteasa de aproximadamente 40 kDa. *Conclusiones:* Los resultados de este estudio confirman que las conidias así como el extracto bruto del hongo *M. thaumasium* puede ser usado para controlar *A. vasorum* L_1.

El perfil proteolítico sugirió la presencia de una proteasa (Mt1) de aproximadamente 40 kDa que en el futuro se pueden utilizar en el control biológico de L_1 de este nematodo.

Palabras clave: hongos nematófagos; *Angiostrongylus vasorum*; *Monacrosporium thaumasium*; proteasa.

Introduction

Nematophagous fungi have been successfully used on in vitro studies of biological control. These organisms called "eaters of helminths" stand out for their availability and easy maintenance under laboratory conditions. However, after many years, yet, little is known about the real mechanism of their infection versus nematodes (Larsen, 1999; Araújo et al. 2004). In this context, researches are more and more performed about the production of primary metabolites, such as proteolytic enzymes, which have been studied in assays biological of nematicidal activity (Braga et al., 2012; Soares et al., 2012). On the other hand, nematophagous fungi has been pointed out, especially due to the promising results observed in laboratory conditions through interaction fungi versus first-stage larvae of *Angiostrongylus vasorum* (Braga et al. 2009).

Species of the genus *Angiostrongylus* (*A. costaricensis*, *A. cantonensis* and *A. vasorum*) can cause problems for the definitive host (dogs) and eventually parasitize man (Ash, 1970). The literature has reported some work that have aimed seek alternative measures, such as biological control, to be used in combat of angiostrongyliasis.

The role of the dog as a reservoir and environmental disseminator of potentially zoonotic parasites has been reported long ago. Moreover, it is known that in many places this important aspect of transmission of zoonotic agents is present, especially in

poorer regions of Asia, South America, Africa and Australia (Traub et al. 2005). In Brazil, Oliveira-Siqueira et al. (2002) mention that the risk of human infection through contaminated faeces of dogs may be significantly higher than in developed countries where a similar prevalence is observed. In this context, Mota et al. (2003) mention that the faecal environment is the place favorable for the development of helminths that parasitize domestic animals and may also be zoonotic, since in this environment, most genera of helminth passes from the egg stage to infective larvae stage. On the other hand, there is a need for studies aimed at discovering the action of substances produced by these fungi in environments that mimic the natural condition of infection, for example, contaminated faeces, so they can eventually be used in environmental control of larvae of this nematoda. Among those substances, stands out the use of proteases produced by nematophagous fungi (Braga et al. 2011a).

The aim of this work was to study the proteolytic profile of the nematophagous fungus *Monacrosporium thaumasium* and its activity against *A. vasorum* larvae.

Materials and methods

Organisms

Positives faeces of dogs were used for *A. vasorum* previously maintained in the Parasitology Department, Federal University of Minas Gerais (Lima et al. 1985).

To obtain crude extract, it was used an isolate of the nematophagous fungus *M. thaumasium* (NF34a) originated from soil in Brazil and maintained through continuous transfer to solid culture media (corn meal agar 2%) in the Laboratory of the Department of Veterinary Parasitology, Federal University of Viçosa. For the production of crude extract, mycelia of this fungus were obtained by transferring the culture discs (about 5 mm in diameter) to flasks previously autoclaved and containing 50 ml of liquid

medium. The liquid medium was composed of: glucose (10 g/l), casein (10 g/l), K_2HPO_4 (5.0 g/l), $MgSO_4$ (0.10 g/l), $ZnSO_4$ (0.0050 g/l); $FeSO_4$ (0.001 g/l) e $CuSO_4$ (0,50 mg/l). Next, the flasks containing the fungal inoculum grown in shaker under agitation of 120 X g and after six days, the supernatant was collected and filtered using Whatman n^o 1 filter paper at 4 °C. The methodology for production and obtaining of the crude extract was based on the work of Braga et al. (2011a).

Assays

The *in vitro* test was carried out through two assays (A and B). In assay A, 1000 conidia of the fungus N34a were added in positive coprocultures for *A. vasorum*, constituting the treated group. The control group was constituted by coprocultures of positive faeces, without fungus. In assay B, 5 ml of crude extract produced by NF34a were added to coprocultures constituting the treated group and coprocultures with only the addition of distilled water (5 ml) constituted the control group. In both assays, coprocultures were incubated at 25 °C in the dark for 8 days. Six replicates were carried for each assay.

At the end of this period were obtained first-stage larvae (L_1) using the modified method of Baermann in both assays (A and B) and the total amount of these was obtained by simple rule of three (Barçante et al. 2003). Then, data were interpreted by analysis of variance and the efficiency of predation of L_1 in relation to the control groups was assessed by the Tukey test at 1% probability (Ayres et al. 2003). Subsequently, the average percentage reduction of larvae was calculated according to the following equation:

$$\%\text{Reduction} = \left(\frac{\bar{x} \text{ of control larvae} - \bar{x} \text{ of treatment larvae}}{\bar{x} \text{ of control larvae}}\right) \times 100$$

Proteolytic profile

Next, the proteolytic profile of crude extract of the nematophagous fungus *M. thaumasium* (NF34a) was revealed by performing a zymogram, using casein as substrate (Casein-SDS-PAGE), as described by Hummel et al. (1996) with some modifications. The action of the enzyme can be observed by the formation of white halos.

Results

At the end of eight days in assays A and B, it was demonstrated that the conidia and the crude extract of *M. thaumasium* (NF34a) were effective in reducing the number of *A. vasorum* L_1. In addition, at the end of the assays was noted a statistical difference ($p < 0.01$) between the means of larvae recovered from the treated groups compared to control groups with the following percentages: 40.0% and 52.0% for the assays A and B, respectively. Via the zymogram performed, it was revealed the proteolytic profile of crude extract produced by NF34, Figure 1, where can be observed the presence of a halo of digestion of casein, revealing the presence of a protease in height of approximately 40 kDa.

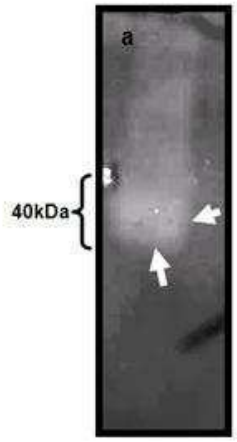

Figure 1. Proteolytic profile of the crude extract produced by the fungus *Monacrosporium thaumasium* (NF34a). White arrows: protease with molecular weight of approximately 40 KDa.

Discussion

The soil contaminated with infective stages (eggs and or larvae) has been appointed as one of major source of contamination by geohelminths (Gortari et al. 2007). Fungal structures, such as conidia has proven to be effective in controlled trials against *A. vasorum* L_1 (Braga et al. 2009,2011b). On the other hand, the use of crude extract is also a artifice, although recent, that has been used to combat eggs and/or larvae of geohelminths (Braga et al 2011b, 2011c). In the present work, the faecal environment was mimetized by use of coprocultures and the results were very promising.

In assay A, conidia of the fungus NF34 poured in the coprocultures decreased the number of L_1 recovered (40.0%). This result is interesting and confirms previous reports of Braga et al. (2009) about the predatory activity of conidia of this fungus after the interaction in culture medium containing agar-water and *A. vasorum* L_1 at the end of seven days (74.5%). However, some comparisons can be performed between the

respective works: (1) in this study were utilized coprocultures (poor medium) to mimic the real condition of contamination, while in the work cited above, was used the agar-water which although is also poor in nutrients probably gives a larger pattern of control, especially in relation to contamination by other agents; (2) the agar-water medium routinely used in laboratory tests has not yet presented the results achieved under natural conditions, being perhaps this one important information for the use of faecal material.

In relation to assay B, the results obtained can be compared also with the same report[5] of the use of crude extract of the predatory fungus *Duddingtonia flagrans* on *A. vasorum* L_1. However, even *M. thaumasium* belonging to the same group of fungi (predators), this information is important because the concentrated crude extract of *D. flagrans* was evaluated directly on the L_1 in a short period of time, 24 and 48 h, demonstrating a percentage reduction of 53.5% and 71.3%, respectively. In the present work, the crude extract of NF34 showed the percentage of reduction of 52.0% in recovery of L_1 obtained from coprocultures treated at the end of eight days.

This result suggests that possibly the crude extracts produced by predatory nematophagous fungi contains hydrolytic enzymes such as proteases, which maintains its biological activity even under non-optimal pH, temperature, humidity and salt concentrations. On the other hand, the zymogram demonstrated a proteolytic profile of the crude extract of NF34a that points to the presence of one protease with molecular weight similar to the proteases described from predatory nematophagous fungi (Huang et al. 2004). Moreover, the zymogram suggested the presence of one protease (Mt1) of approximately 40kDa, which is in according to the recent work of Soares et al. (2012). These results may eventually contribute to the discovery of new methodologies that can help in environmental decontamination of geohelminths.

The results of this work confirm that the conidia as well as the crude extract of the fungus *M. thaumasium* may be used to control *A. vasorum* L_1. The proteolytic profile suggested the presence of one protease (Mt1) of approximately 40 kDa that in the future may be used in biological control of L_1 of this nematode.

Authors' declaration

We are taking the opportunity to declare that: (a) the contents of the article are original and they were not published previously; (b) there is no conflict of interests, related to financial aspects; (c) all the authors have read and approved this manuscript.

Conflicts of interest

All authors declare no conflict of interest.

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CAPÍTULO 3

Proteolytic activity of the nematophagous fungus *Arthrobotrys sinensis* on *Angiostrongylus vasorum* larvae

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Proteolytic activity of the nematophagous fungus *Arthrobotrys sinensis* on *Angiostrongylus vasorum* larvae

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Abstract

Abstract

Background: The predatory nematophagous fungus *Arthrobotrys sinensis* (SF53) produces three proteases with nematicidal activity when grown on solid media culture. However, the proteolytic profile produced by this fungus, when grown in liquid culture medium remains unknown.

Findings: Thus, the objective of this work was to evaluate the production of proteases from nematophagous fungus *Arthrobotrys sinensis* in liquid medium and its nematicidal activity on first stage larvae of *A. vasorum.* Proteases were obtained in its crude form, using Whatman no.1 filter paper, followed by centrifugation for 5 min at 10 X g and 4 °C. A zymogram was performed with co-polymerized casein in an acrylamide gel as substrate. An *in vitro* assay to evaluate the nematicidal action of the proteases of *A. sinensis* (SF53) produced in liquid medium on *A. vasorum* L_1 was conducted. By the analysis of the zymogram, it was observed a single halo at the beginning of digestion of the gel, suggesting that the three proteases of SF53 are produced in an enzymatic complex of large molecular weight. Regarding nematicidal activity, within 24 hours, the proteases produced in liquid medium of *A. sinensis* (SF53) showed a percentage reduction of 64% on the number of L_1 of *A. vasorum*.

Conclusion: In the present work, it is suggested that the three proteases of SF53 are produced in an enzymatic complex and was also demonstrated that these enzymes were effective in destroying *A. vasorum* L_1.

Keywords: Nematophagous fungi, *Angiostrongylus vasorum*, protease

Findings

Background

Angiostrongylus vasorum (Baillet, 1866) Kamensky, 1905, is a protostrongylidae parasite nematode of domestic dogs and wild canids, which causes angiostrongylosis, disease important in public health. In this aspect, stands out the presence of free larvae in the environment and thus the possibility of human infection, since other parasites of the genus *Angiostrongylus* are proven zoonotic. In dogs, the disease is associated with the occurrence of cough, dyspnea, exercise intolerance, weight loss, vomiting, neurological signs, heart failure and death. The infection of dog may occur when ingesting 1) infected paratenic hosts, such as frogs and small mammals, 2) infected intermediate hosts (molluscs) of the genera *Biomphalaria*, *Physa* and among others, 3) or food or water contaminated with free infective larvae in the environment [1, 2].

Despite some successful cases, currently the control of this parasite has been associated with use of anthelmintics that although routinely used, do not act very well on the parasite in the definitive host [3]. Moreover, angiostrongylosis rarely develops acutely and in this sense the clinical signs are perceived later, allowing a continuous environmental dispersion of the parasite through the feces of infected dogs. Some authors have shown that the use of complementary measures to combat helminthoses that complete their development in the environment can be used as tools of control [4, 5]. Thus, the use of nematophagous fungi is cited here. These organisms use mechanical devices such as modified hyphae (traps) and enzymatic artifices to overcome

the nematode larvae (production of hydrolytic enzymes, especially proteases) [6].

According to Soares and colleagues [5], one of the promising genera of nematophagous fungi is *Monacrosporium*. Those authors have developed some works that demonstrate their nematicidal activity. In this context, in a recent study, Soares and colleagues [7] showed that the predatory nematophagous fungus *M. sinense* (SF53) produces three proteases with nematicidal activity when grown on solid media culture. It is suggested that these extracellular proteases are important at various stages of infection, such as release of nutrients for growth of the microorganism penetration of the cuticle and digestion of the host tissue. However, the production of an enzymatic complex by this fungus remains unclear.

Thus, the objective of this work was to evaluate the production of proteases from nematophagous fungus *Arthrobotrys sinensis* in liquid medium and its nematicidal activity on first stage larvae of *A. vasorum*.

Materials

The nematophagous fungus *A. sinensis*, isolate SF53, was used for the production of proteases in liquid medium. This isolate is derived from Brazilian soil and has been kept under laboratory conditions through continuous transfer to solid medium. The fungus was cultivated for 10 days in the dark. Then fungal mycelia were transferred to previously autoclaved flasks containing 50 ml of liquid medium composed of (in grams per liter): glucose, 10; yeast extract, 10; K_2HPO_4, 5; $MgSO_4$, 0.10; $ZnSO_4$, 0.005; $FeSO_4$, 0.001; $CuSO_4$, 0.0005. The inoculum has grown in shaken flasks at 120 X g. After 6 days, proteases were

obtained in its crude form, using Whatman no.1 filter paper, followed by centrifugation for 5 min at 10 X g and 4 ° C [8].

In this study, the strain of *A. vasorum* used has been maintained by the Department of Parasitology, Federal University of Minas Gerais and it is originated from naturally infected dogs, from the city of Caratinga, Minas Gerais [9]. For its obtaining, faeces of infected dogs were collected and placed in a modified Baermann apparatus for the recovery of L_1. The faeces remained in the apparatus for 12 hours. After this period, the tube was removed, centrifuged at 200 X g for 2 min, the supernatant was discarded and the pellet containing the *A. vasorum* L_1 was resuspended in 5 ml of 0.85% NaCl. The content present in the tube was homogenized, and from this three aliquots were taken of 10 µL, distributed in glass plate of 7.5 x 2.5 cm. The larvae were counted using a stereomicroscope at increase of 25x [1].

The proteolytic activity was measured by the method of Soares and colleagues [7]. A standard curve of tyrosine was built for the quantification of enzyme activity. One unit of protease was defined as the amount of enzyme required to liberate 1.0 µg of tyrosine per minute under the assay conditions.

A zymogram with co-polymerized casein in an acrylamide gel [10] as substrate (casein-SDS-PAGE) was performed as described by Soares and colleagues [7]. The proteolytic activity was observed by the formation of white halos. The halos of digestion were excised and analyzed by SDS-PAGE (Laemmli, 1970) in order to verify the presence of enzymes.

An *in vitro* assay was conducted to evaluate the nematicidal action of the proteases of *A. sinensis* (SF53) produced in liquid medium on *A. vasorum* L_1 following the methodology of Soares and colleagues [8]. The data obtained in

this experiment were interpreted by analysis of variance in significance levels of 1 and 5% probability. The efficiency of L_1 predation compared to control was assessed by the Tukey test at 1% probability [11]. Subsequently, the percentage reduction of average larvae (L_1) was calculated according to the following equation:

$$\% = \frac{(\bar{x}\,L_1 \text{ recovered from control} - \bar{x}\,L_1 \text{ recovered from treatment})}{\bar{x}\ L_1 \text{ recovered from control}} \times 100$$

Results and Discussion

It was observed that the fungus *A. sinensis (SF53)* has produced proteases, when grown in an inducer liquid medium. However, proteolytic activity (15.78 U/mL) was lower than that obtained when the same fungus was grown on solid culture medium (38.0 U/mL) [7]. Moreover, a single halo was observed at the beginning of digestion of the gel, suggesting that the three proteases of SF53 are produced in an enzymatic complex of large molecular weight (Fig. 1). When grown on solid culture medium, three evident halos were observed in the zymogram of the same fungus [7]. Probably this difference is due to the enzymatic extraction by stirring, a more brute technique, in the case of solid medium, which may have ruptured the enzymatic complex, “releasing” each protease.

Figure 1. Zymogram of the proteases produced by *Arthrobotrys sinensis (SF53)* in liquid medium. Through analysis of the zymograms, a single halo of digestion at the beginning of the gel was observed, suggesting that the three proteases of SF53 are produced in an enzymatic complex of large molecular weight.

Regarding nematicidal activity, within 24 hours, the proteases produced in liquid medium of *A. sinensis (SF53)* showed a percentage reduction of 64.3% on the L_1 of *A. vasorum*. Also, difference ($p < 0.01$) was observed in nematicidal action in relation to larvae present in the control group, in the same studied interval.

The predatory activity of fungi of *Monacrosporium* genus has been tested on *A. vasorum* L_1. However, only one early work [12] had demonstrated its capture and subsequent *in vitro* destruction, in culture medium WA2%. Braga and colleagues [12] demonstrated that isolate SF53 was effective ($p < 0.05$) in the capture and destruction of *A. vasorum* L_1 under laboratory conditions, registering at the end of seven days the percentage reduction of 74.2%. However, in the present work, it was noted that the obtained percentage reduction was 64.3%, what is interesting from a biological point of view, since only enzyme was used (no fungi).

Furthermore, our results suggest that the nematicidal activity of SF53 was due to the action of enzymes on the cuticle of the L_1 of *A. vasorum*, since the cuticles of larvae are especially rich in proteic components that hinder the action of antagonist organisms (Fig. 2). Accordingly, another work developed by the present group showed that the use of crude enzyme extract of nematophagous fungus on *Ancylostoma caninum* L_3 (a geohelminth) showed good efficacy [13]. In that work, it was observed the hydrolysis of the cuticle by enzymatic action, which also has acted inside the nematode, causing its destruction.

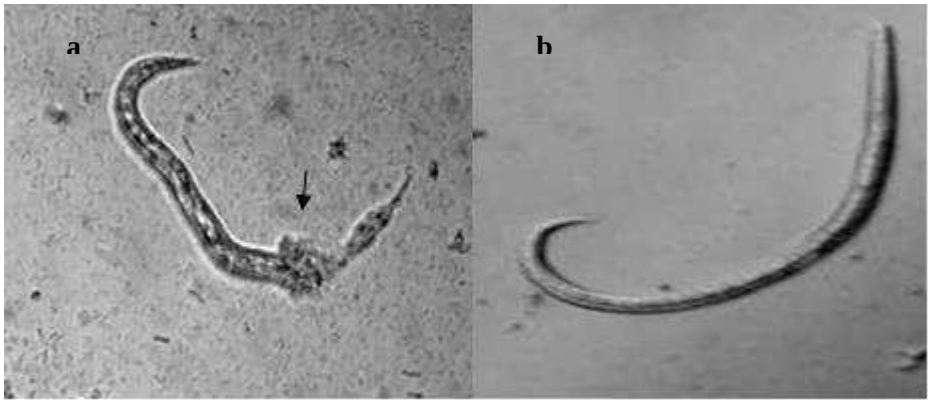

Figure 2 (a-b). Photomicroscopy of nematicidal activity of proteases from nematophagous fungus *Arthrobotrys sinensis* (SF53) on first stage larvae of *Angiostrongylus vasorum* after 24 hours (treated group (a) and control group (b)).

Soares and colleagues [7] reported that extracellular proteases are an important virulence factor for the species *M. sinense*. Its nematicidal activity was also evaluated on *Panagrellus redivivus* larvae (free-living nematode), and

at the end of the experiment the average percentage was 79% of reduction in the number of recovered larvae. In the present work, another enzyme production was tested using liquid culture medium, and the results were interesting both for proteolytic and nematicidal activity. Furthermore, it is also suggested that the use of *A. vasorum* can probably contribute to further research about its control.

Three proteases were produced in both cases (solid and liquid medium), however, probably because of differences in the extraction process (much more gentle in the case of liquid medium, using only filtration and centrifugation, than in the case of the solid medium in which there is an intense mechanical agitation), we can find at beginning of the gel a halo that suggests the presence of an enzymatic complex of large molecular mass, which has failed to migrate into the gel, due to this large mass.

In the present work, three proteases of the isolate SF53 were produced in an enzymatic complex and was also demonstrated that these enzymes were effective in destroying *A. vasorum* L_1 under laboratory conditions.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Queiroz, JH; Lima,WS; Araújo, JV contributed in the designing and coordinating the experiment and also in the preparation and revision of the manuscript. Soares, FEF and Braga, FR carried out the experiments. All authors read and approved the final manuscript.

Authors' information

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CAPÍTULO 4

Action of the nematophagous fungus *Pochonia chlamydosporia* on *Dioctophyma renale* eggs

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Action of the nematophagous fungus *Pochonia chlamydosporia* on *Dioctophyma renale* eggs

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Abstract

The ovicidal action of the nematophagous fungus *Pochonia chlamydosporia* (VC4) was evaluated on *D. renale* eggs under laboratory conditions (Assay A). Next, the enzymatic action of proteases and chitinases produced by *P. chlamydosporia* (VC4) was evaluated on *D. renale* eggs, under laboratory conditions (Assay B). At the end of the experiment, there was difference ($p<0.01$) in the destruction of eggs in the four concentrations tested in relation to control group at each interval studied. On the other hand, no difference was observed ($p>0.01$) among the concentrations in the destruction of eggs. However, there was a trend of increasing mortality with increased concentration. Then (assay B), it was observed that in the 24-hour interval, the proteases and chitinases of *P. chlamydosporia* (VC4), either individually or together, have caused a significant percentage reduction ($p <0.01$) on the number of viable eggs of *D. renale*, compared to control, with the following reduction values: 27.8% (proteases), 29.4% (chitinases) and 43.4% (proteases + chitinases). Thus, the constant search for alternatives that may help combat the various infectious forms (or eggs and larvae) of potentially zoonotic nematodes are important, as the use of fungi destroyers of eggs. Therefore, it is suggested the application of *P. chlamydosporia*, in biological control of eggs from that nematode.

Keywords: ovicidal fungus, dioctophymosis, protease, chitinase, biological control

1. Introduction

The ovicidal effects of the fungus *Pochonia chlamydosporia* on eggs of different genera of geohelminths and biohelminths have shown promising results under laboratory conditions, which in the future can be extrapolated to natural conditions (Tavela et al., 2013; Braga et al., 2012).

The giant kidney worm, *Dioctophyma renale*, is the largest nematode known (Bowman et al., 2006). Its complex life cycle requires an aquatic oligochaete or annelid as an intermediate host. Fish and frogs are paratenic hosts that serve to infect the definitive hosts (DH) (Mace & Anderson, 1975)., Wild carnivores such as mustelids, canids, and some domesticated animals are known definitive hosts, with sporadic reports in atypical hosts which include humans (Woodhead, 1950). The DH is infected from ingestion of meat of raw fish and undercooked frogs. In these animals, the pathogenesis is the destruction of the kidney; however, except for some situations it may be asymptomatic even when one of the kidneys is completely destroyed (Urquhart et al., 1998).

Eggs of *D. renale* are eliminated through urine of the dog and its development occurs in the external environment. In addition, its shell thick and wrinkled confers resistance in the environment for years. The literature, however, points out that the control and prevention of dioctofimosis are based on the elimination of raw fish from the diet (Urquhart et al., 1998; Birchard & Sherding, 2003). However, measures such as biological control that can help decrease egg concentrations may aid in the control of this zoonosis. The present study aimed to evaluate the action of the nematophagous fungus *Pochonia chlamydosporia* on *Dioctophyma renale* eggs.

2. Material and methods

2.1. Fungi and culture medium

The isolate VC4 of the nematophagous fungus *P. chlamydosporia* originated from soil of Brazil has been maintained by continuous transfer to solid media culture in the Laboratory of Parasitology of the Department of Veterinary of the Federal University of Viçosa. The fungus was cultured in Petri dishes containing 20 mL of medium YPSSA (yeast extract, 4 g: K_2HPO_4 1 g: $MgSO_4$, 0.5 g: soluble starch, 20 g: agar, 20 g: sufficient water to 1 L of solution) at 25 °C for 28 days in absence of light. After this period, the surface of the plates was washed with 10 mL of distilled water with a brushand passed through a sieve to eliminate mycelia fragments. Spores recovered were quantified in ten counts in a Neubauer chamber and qualified (chlamydospores) in aliquots of 10μL (Gams & Zare, 2001). The spores were then diluted to obtain final concentrations of 500, 1000, 1500 and 2000 spores/mL.

For the production of enzymes, two different culture media were inoculated with spores of *P. chlamydosporia* (VC4). The culture medium used for the production of protease contained: 0.3 g/L NaCl, 0.3 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L K_2HPO_4 and 0.2 g/L yeast extract and 0.2% casein, as described by Esteves et al. (2009), modified. One solid culture medium was used for the production of chitinase, with wheat bran (5 g) supplemented with 1% chitin as substrate, and 2.5 mL of liquid minimal medium, in grams per liter K_2HPO_4 (5.0), $MgSO_4$ (0.10), $ZnSO_4$ (0.0050), $FeSO_4$ (0.001) and $CuSO_4$ (0.0050) (Braga et al., 2013). The extraction of the chitinases was performed according to Soares et al. (2013). The clear supernatant was used for the assays.

2.2. *Dioctophyma renale* eggs

D. renale eggs were obtained by dissection from the uterus of an adult female specimen that was recovered during the course of a total nefrectomia of a young parasitized dog (Fig. 1 a-b) at the clinical sector and surgery of Department of Veterinary of the Federal University of Viçosa. The identification of adult specimens and eggs was based on description of Urquhart et al. (1998). The eggs were washed 10x in distilled water by centrifugation at 1000 g for 5 minutes each time, discarding the supernatant after each centrifugation.

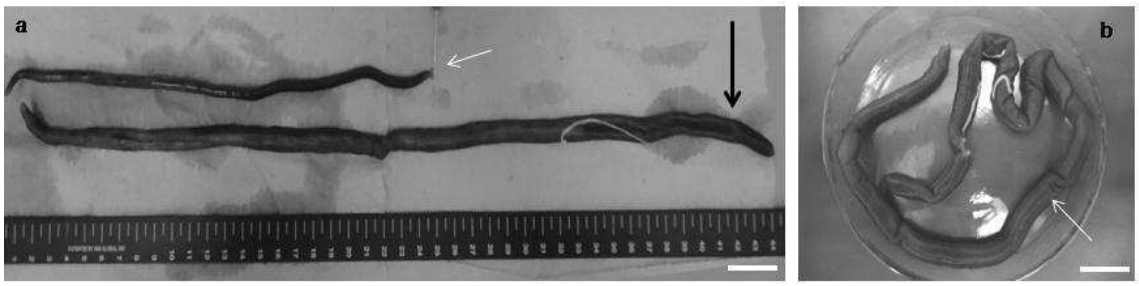

Fig. 1ab – Specimens of the nematode *Dioctophyma renale*, male (white arrow) and female (black arrow), recovered during the realization of a total nefrectomia of a young dog parasitized in the sector of clinical and surgery of Department of Veterinary of the University of Viçosa. Bars: A – 4 cm; B – 2.6 cm.

2.3. Experimental assays

The ovicidal action of *P. chlamydosporia* (VC4) was evaluated on *D. renale* eggs under laboratory conditions (Assay A). Next, the enzymatic action of proteases and chitinases produced by *P. chlamydosporia* (VC4) was evaluated on *D. renale* eggs, under laboratory conditions (Assay B).

2.4. Assay A

D. renale eggs were transferred over the surface of 9 cm diam Petri containing 2% water-agar (2% WA) medium at the following concentrations of chlamydospores (500, 1000 1500 and 2000 spores/mL). Six replicates were performed for each group. In the treatments each plate contained one thousand eggs of *D. renale* with only one of the concentrations and the control group with only one thousand eggs. At 3, 5 and 7 days, 100 eggs were taken from each plate of the treatments and of control without chlamydospores according to the technique described by Araújo et al. (1995), then evaluated at total magnification of 400x according to Lysek et al. (1982): type 1, lytic effect without morphological damage to eggshell, where hyphae are observed adhering to the shell, type 2, lytic effect with morphological alteration of the shell and embryo egg without hyphal penetration through the shell and type 3, lytic effect with morphological alteration of embryo and shell, in addition of hyphal penetration and internal colonization of the egg. The data for each interval studied were submitted to Friedman's nonparametric test with 1% probability (Ayres et al., 2003).

2.5. Assay B

Four groups were formed in sterile tubes, three treatment groups, one group treated with only VC4 proteases, one group treated with only VC4 chitinases, one group treated with both classes of enzymes (proteases and chitinases) and one control group (no enzyme). To avoid protease activity in the group treated only with chitinases, the protease inhibitor PMSF (phenylmethylsulfonyl fluoride) was used. Six replicates were performed for each group. One hundred *D. renale* eggs were transferred into sterile tubes containing 20 µl of enzyme for each treated group. The control group consisted of one hundred *D. renale* eggs. The sterile tubes were incubated at 28 °C in the dark for 24

hours. After 24 hours, the total number of *D. renale* intact eggs present in each of the treated and control tubes was calculated according to the modified method described by Braga et al. (2012).

The data obtained in this test were interpreted by analysis of variance with significance levels of 1 and 5% probability. The efficiency of destruction of *D. renale* eggs compared to the control was assessed by the Tukey test at 1% probability. Subsequently, the percentage reduction of the average number of eggs was calculated according to the following equation:

$$\% = \frac{(\bar{x} \text{ of intact eggs from control} - \bar{x} \text{ of intact eggs from treatment})}{\bar{x} \text{ of intact eggs from control}} \times 100$$

3. Results

The percentage results for the effects of types 1, 2 and 3 to 3, 5 and 7 days of interaction of different concentrations of chlamydospores (Assay A) are shown in figure 2.

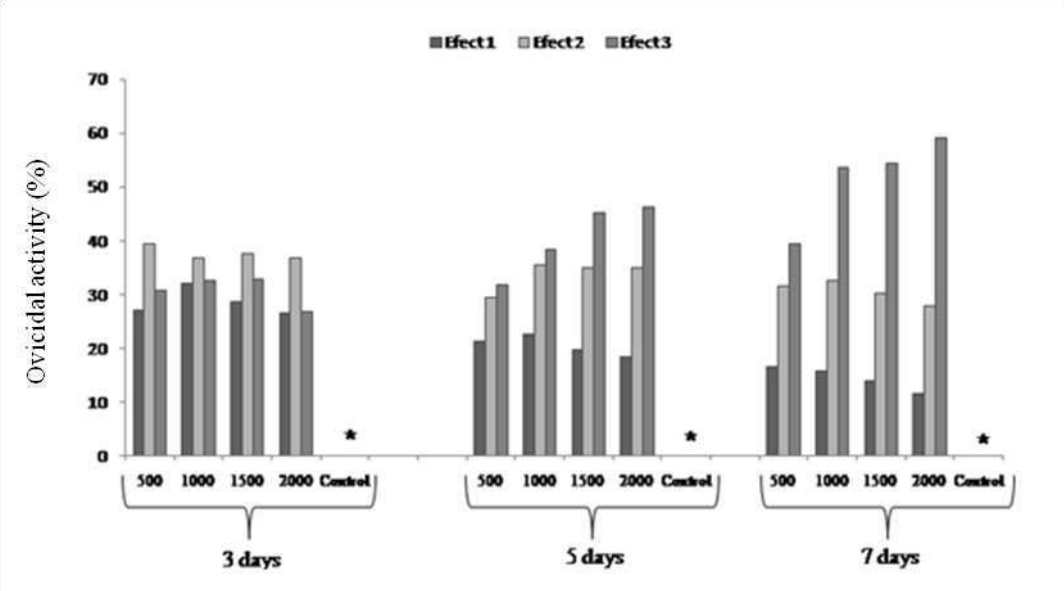

Fig. 2 – Percentages of the ovicidal activity for the effects of types 1, 2 and 3 to 3, 5 and 7 days of interaction of chlamydospores of the fungus *Pochonia chlamydosporia* (VC4) at different concentrations (500, 1000, 1500 and 2000) on eggs of *Dioctophyma renale* and the control group. The asterisk denotes a difference ($p<0.01$).

At the end of the experiment, there was difference ($p<0.01$) in the destruction of eggs (effect of type 3) in the four concentrations tested in relation to control group at each interval studied. On the other hand, among the concentrations no difference was observed ($p>0.01$) in the destruction of eggs. However, there was a trend of increasing mortality with increased concentration. Regarding the visualization of the eggs destroyed (effect of type 3), these were observed by light microscopy at 40x objective (Fig. 3 a-e).

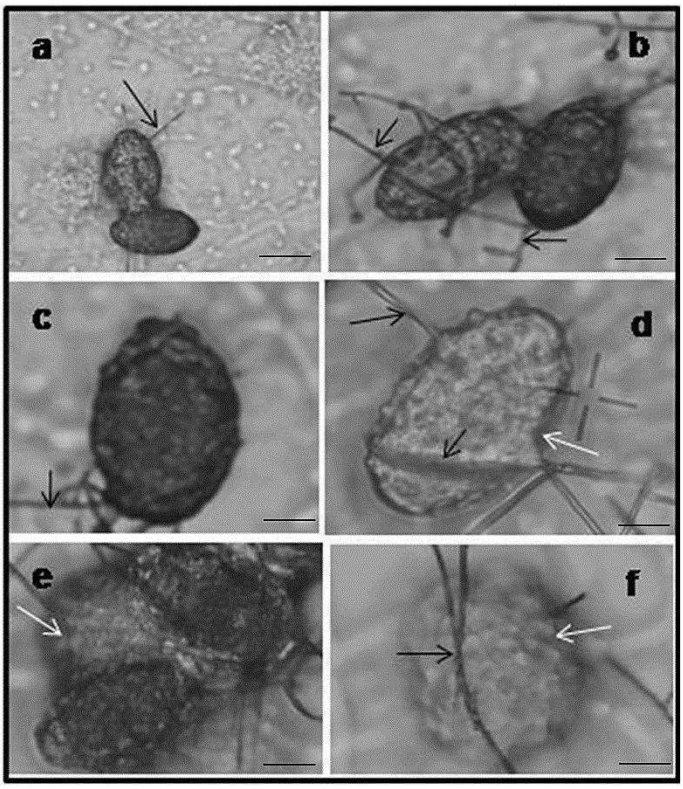

Fig. 3 a-f - Hyphae of the fungus *Pochonia chlamydosporia*, a-d (black arrow), and the eggs of *Dioctophyma renale* destroyed, e-f (white arrow) at the end of the experiment. Light microscope, 40x objective. Bars: (a) 67 µm. (b) 201 µm; (c) 201 µm; (d) 301 µm; (e) 268 µm and (f) 235 µm.

In assay B, in the 24-hour interval, the proteases and chitinases of *P. chlamydosporia* (VC4), either individually or together, caused a significant percentage reduction ($p < 0.01$) on the number of viable eggs of *D. renale*, compared to control, with the following reduction values: 28% (proteases), 29.4% (chitinases) and 43% (proteases + chitinases). Significant differences waere observed ($p<0.05$) between the group treated with proteases in relation to the group treated with proteases + chitinases. Figure 4 depicts the destruction of eggs after previous contact with both classes of enzymes.

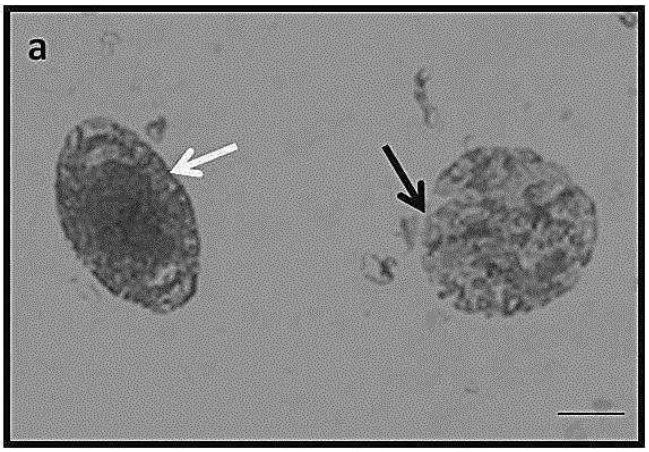

Fig. 4 - *Dioctophyma renale* egg destroyed (black arrow) after previous contact with both classes of enzymes (chitinases and proteases) produced by *Pochonia chlamydosporia.* Intact egg of *Dioctophyma renale* (white arrow). Light microscope, 10x objective. Bars: 168 µm.

4. Discussion

The four concentrations of chlamydospores (500, 1000, 1500 and 2000) were effective in destroying the eggs suggesting that *P. chlamydosporia* could be used in biological control of potentially zoonotic helminths eggs (Araujo et al., 2009). *P. chlamydosporia* has previously been shown to be ovicidal activity against eggs of trematodes, cestodes and nematodes *in vitro* under laboratories conditions (Braga et al., 2007, 2008, Araujo et al., 2009), however, this is the first report of its ovicidal activity against eggs of *D. renale*.

The production of chlamydospores, resistant structure, is one of the main characteristics of a fungus, which possibly could be used in environmental control of parasitic gastrointestinal helminths eggs (Braga et al., 2007). These structures provide the nematophagous fungi the capacity to pass through the gastrointestinal tract of domestic animals and be dispersed in the external environment colonizing it, (Braga et al., 2010). In this context, *P. chlamydosporia* chlamydospores demonstrated effective in

destroying eggs of *Taenia taeniaeformis*, a cestoda parasite of cats and, were efficient in their destruction (Braga et al., 2011). Araujo et al. (2009) demonstrated that isolate VC4 destroyed 2.2, 7 and 8% of *T. saginata* eggs after 5, 10 and 15 days exposure. There are only a few reports on production of enzymes by nematophagous fungi and their larvicidal and/or ovicidal activity (Soares et al., 2013). These, have evaluated the larvicidal activity of *Monacrosporium thaumasium* proteases on *Angiostrongylus vasorum*, a potentially zoonotic nematode. On the other hand, Braga et al. (2011) evaluated the ovicidal activities (type 3 effect) of VC1 and VC4 isolates of *P. chlamydosporia* in a solid medium and the action of *P. chlamydosporia* proteases against eggs of *Ascaridia galli*. In the present work, proteases and chitinases were produced in different culture media. It was observed that the chitinases of *P. chlamydosporia* were significant in destroying eggs of *D. renale*, which suggests further studies to elucidate the mechanism of interaction. In addition, the authors emphasize that this is the first report on the visualization of the destruction of *D. renale* eggs after prior contact with extracellular enzymes produced by *P. chlamydosporia*.

Parasitic infections that can be transmitted by ingestion of products of animal origin are numerous, and in most cases, man may eventually become one of its hosts (Urquhart et al., 1998; Prado & Capuano, 2006; Xhaxhiu et al., 2011). Thus, the constant search for alternatives that may help combat the various infectious forms (or eggs and larvae) of potentially zoonotic nematodes are important, as the use of fungi as destroyers of eggs. Therefore, *P. chlamydosporia*, may have potential in biological control of zoonotic nematodes.

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CAPÍTULO 5

Statistical screening for the chitinase production by nematophagous fungi from *Monacrosporium* genus

Submetido ao periódico “African Journal of Microbiology Research”

Statistical screening for the chitinase production by nematophagous fungi from *Monacrosporium* genus

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Abstract

The aim of this study was to perform the statistical screening of the components of the culture medium for chitinase production by nematophagous fungi *Monacrosporium sinense* (SF53) and *M. thaumasium* (NF34) aiming to identify which of these components were significant and to evaluate the influence of pH on chitinase activity of these fungi. Conidia of these fungi were inoculated in a medium containing wheat bran supplemented with chitin for chitinase production. Chitinase activity was measured through the measure of the reducing sugars using DNS method. The effect of five variables (chitin, colloidal chitin, glucose, sodium nitrate, moisture) on chitinase production was analyzed using Plackett-Burman statistical design. In relation to the isolate NF34, at the levels evaluated, two variables were significant ($p < 0.05$) for chitinase production: chitin and $NaNO_3$. On the other hand, for SF53, at the levels evaluated, only glucose was significant ($p < 0.05$). Regarding the influence of pH on

enzyme activity for *M. thaumasium* the pH value with the highest activity obtained was 5.5. However, for *M. sinense*, the highest activity was observed at pH 8. These data demonstrate that different species of nematophagous fungi may exhibit better enzymatic activities and consequently, better predatory activities at different environmental conditions.

Keywords: nematophagous fungi; chitinase; Plackett-Burman

Introduction

According to Li (2006) the second most abundant polymer in the planet Earth is chitin, a homopolymer composed of units of N-acetyl-β (1-4)-D-glucosamine (GlcNAc). In general biological terms, this polymer is essential for body constituition of a number of organisms and, among these: cuticles and membranes of insects, cell walls of fungi and helminth eggshells (Gortari and Hours, 2008). Moreover, it has been demonstrated that the destruction of the exoskeleton of arthropods can be done in a biological way by using chitinases that certain fungi can produce (St et al., 1996; Bogus et al., 2005; Braga et al., 2013).

Chitinases (EC 3.2.1.14) are glycosidases that hydrolyze β (1-4) linkages of N-acetylglucosamine residues of chitin. As mentioned earlier, these enzymes can be produced by nematophagous fungi as an important virulence factor in the infection process of "target organisms" (Braga and Araújo, 2014). Several chitinases with nematicidal action of these fungi have been identified in recent years, however, little is known about which nutritional factors from the culture medium are significant for its mass production (Tikhonov et al., 2002; Khan et al., 2004; Gan et al., 2007; Yang et al., 2010; Yang et al., 2013).

In order to quickly clarify the influence of certain nutritional factors in the culture medium for the production of chitinase is necessary to use a mathematical technique to design experiments, reducing the amount of assays and also time saving (Ma et al., 2012). In this sense, Plackett-Burman design (Plackett and Burman, 1946) is a useful and efficient statistical approach to evaluate the effect of various factors on a specific response, and it has proved effective in studies involving the production of enzymes from nematophagous fungi (Braga et al., 2009; Soares et al., 2013a,b).

Thus, the aim of this study was to perform the statistical screening of the components of the culture medium for the production of chitinase by two isolates of nematophagous fungi from the *Monacrosporium* genus aiming to identify which of these components were significant and to evaluate the influence of pH on chitinase activity of these fungi.

Materials and Methods

Organisms

Two isolates of nematophagous fungi from the *Monacrosporium* genus (*M. sinense* (SF53) and *M. thaumasium* (NF34)) derived from mycology collection of the Laboratory of Parasitology of the Veterinary Department of the Federal University of Viçosa, Minas Gerais, Brazil were used. These isolates have been kept in test tubes at 4 °C containing 2% corn-meal-agar (2% CMA) in the dark. From these test tubes, culture dishes were extracted and transferred to Petri dishes containing 2% potato dextrose agar (2% BDA), maintained at 26 °C in the dark for 10 days (Braga et al. 2010).

Solid state fermentation

Conidia of *M. thaumasium* (NF34) and *M. sinense* (SF53) were placed in sterile saline to prepare the inoculum for the fermentation. Wheat bran (5g) (commercially obtained in Viçosa, Minas Gerais, Brazil) supplemented with 1% chitin was used as substrate for the production of chitinase. Erlenmeyer flasks (125 mL) were used as experimental units. The moisture was adjusted to 50% (v / w) using saline solution containing K_2HPO_4 (5,0 g/l) and $MgSO_4$ (0.10 g/l). Each vial was covered with hydrophobic cotton and autoclaved at 121 ° C for 15 min. After cooling, each flask was inoculated with the conidial suspension previously prepared and incubated in a BOD incubator at 28 °C for five days (Braga et al., 2013). After this period, chitinase produced was extracted according to the method of Soares et al. (2010). The clear supernatant was used for the assays.

Enzymatic activity

Chitinase activity was measured through the measure of the reducing sugars using DNS method (Miller, 1959). The assay was composed of: 10 µL of enzyme, 40 µL of sodium acetate buffer 50 mM pH 5.5 and 50 µL of 1% colloidal chitin previously prepared according to the method of Braga et al. (2013), incubated at 50 °C. The reaction was stopped by addition of 100 µL of DNS reagent. The amount of reducing sugars released was determined by recording the absorbance at 540 nm. A control was performed by adding denatured enzyme to the reaction mixture. A standard N-acetylglucosamine curve was constructed by varying its concentration. One unit of chitinase was defined as the amount of enzyme required to liberate 1.0 µmol of N-acetylglucosamine per minute under the assay conditions.

Influence of pH on the chitinase activity

Chitinase activity was determined at different pHs using the following buffers for the respective values of pH: sodium acetate 50 mM (pH 3.5, 4.5 and 5.5) and Tris-HCl 50 mM (pHs 7,0 and 8,0). The test temperature was 50 °C.

Statistical screening

The Plackett-Burmann factorial design was conducted to find which variables were significant for the production of chitinase by nematophagous fungi *M. thaumasium* (NF34) and *M. sinense* (SF53). The experiment was performed in duplicate. The values of activity corresponds to the average of triplicates. Plackett-Burman design can be used for rapid multifactorial screening in order to find the physical and chemical significant factors necessary for high enzyme production (Plackett and Burman, 1946). Each factor was assessed at two levels: high (+1) and low (-1), with five variables (chitin, colloidal chitin, carbon source (glucose), nitrogen source (sodium nitrate - NaNO3), moisture) (Table 1). In this design, it is assumed that the main factors have no interactions. Thus, the multiple regression model of the first order is given by the following equation:

$$Y = \beta_o + \beta_i x_i + \beta_{ii} x_{ii} + \beta_{iii} x_{iii} + \ldots + \beta_n x_n$$

Table 1. High (+1) and low (-1) levels of the five variables (chitin, colloidal chitin, carbon source (glucose), nitrogen source (NaNO3), moisture) in Plackett-Burman statistical design

Variables	High level (+1)	Low level (-1)
Chitin	1.5	0.5
Colloidal chitin	1	0
Glucose	1	0
$NaNO_3$	1	0
Moisture	350	150

Where Y is the response (chitinase activity in U/mL), βo is the intercept of the model and βi to βn are the coefficients of the response values, and xi is the level of the independent variable. Minitab 15 software was used for analysis of Plackett-Burman statistical design. Data were analyzed by analysis of variance (ANOVA) with statistical significance level of $p < 0.05$.

Results

The effect of five variables (chitin, colloidal chitin, glucose, sodium nitrate, moisture) on chitinase production by *M. thaumasium* (NF34) and *M. sinense* (SF53) was analyzed using Plackett-Burman statistical design (Tables 2 and 3).

Table 2. Matrix of the Plackett-Burman experimental design of chitinase production (U/ml) by the nematophagous fungus *Monacrosporium thaumasium* (NF34)

Runs	Chitin	Colloidal chitin	Glucose	$NaNO_3$	Moisture	Activity (U/ml)
1	1.0	0	1	0	150	0.405
2	1.0	1	0	1	150	0.138
3	0.5	1	1	0	300	1.24
4	1.0	0	1	1	150	0
5	1.0	1	0	1	300	0
6	1.0	1	1	0	300	0.319
7	0.5	1	1	1	150	0.233
8	0.5	0	1	1	300	0.324
9	0.5	0	0	1	300	0.144
10	1.0	0	0	0	300	0.152
11	0.5	1	0	0	150	1.04
12	0.5	0	0	0	150	0.432

Table 3. Matrix of the Plackett-Burman experimental design of chitinase production (U/ml) by the nematophagous fungus *Monacrosporium sinense* (SF53)

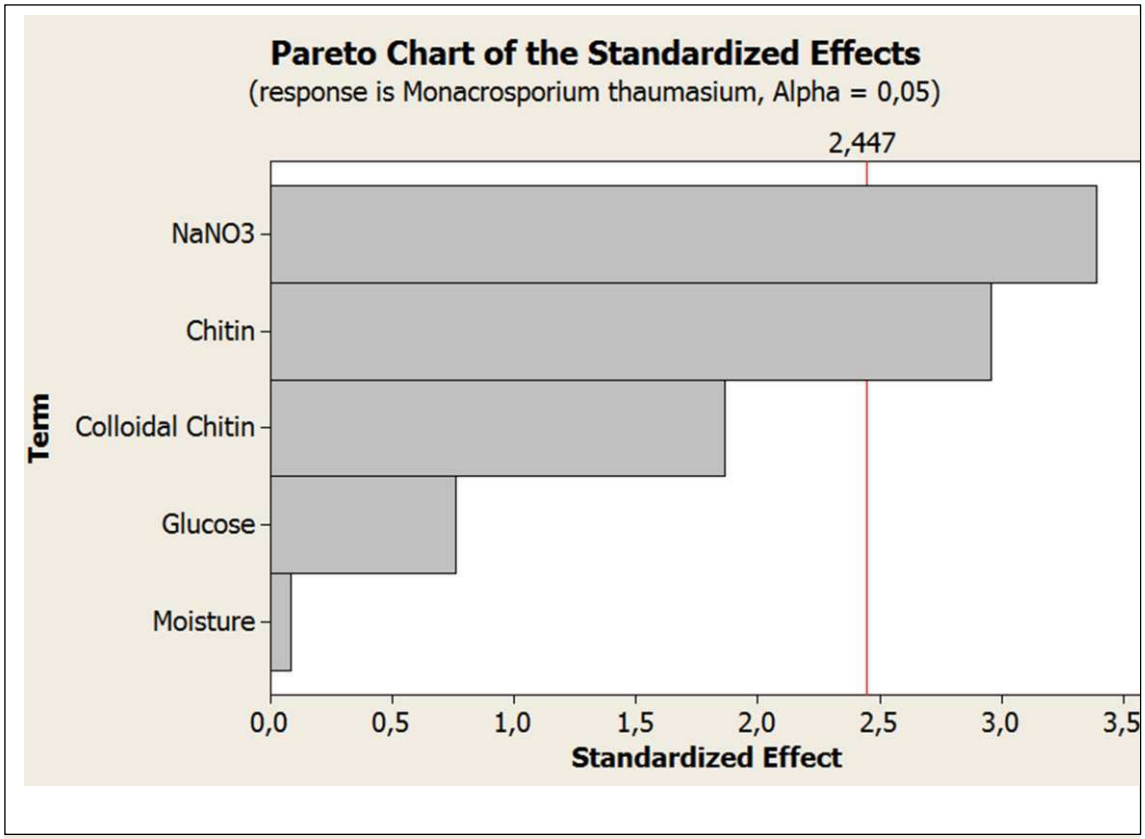
Runs	Chitin	Colloidal chitin	Glucose	$NaNO_3$	Moisture	Activity (U/ml)
1	1.0	0	1	0	150	0.265
2	1.0	1	0	1	150	0,335
3	0.5	1	1	0	300	0,306
4	1.0	0	1	1	150	0,257
5	1.0	1	0	1	300	0,246
6	1.0	1	1	0	300	0,133
7	0.5	1	1	1	150	0,208
8	0.5	0	1	1	300	0,198
9	0.5	0	0	1	300	0,292
10	1.0	0	0	0	300	0,403
11	0.5	1	0	0	150	0,303
12	0.5	0	0	0	150	0,487

In relation to the isolate NF34 could be seen that, at the levels evaluated, two variables were significant ($p < 0.05$) (Fig. 1A) for chitinase production: chitin and $NaNO_3$ (Table 4).

Table 4. Analysis of the factors studied in the Plackett-Burman statistical design of chitinase production by nematophagous fungus *Monacrosporium thaumasium* (NF34).

	Effect	**P>|*t*|**
Constant	-	0.002
Chitin	-0.3998	0.025
Colloidal chitin	0.2520	0.111
Glucose	0.1017	0.480
$NaNO_3$	-0.4583	0.015
Mositure	-0.0126	0.929

A



B

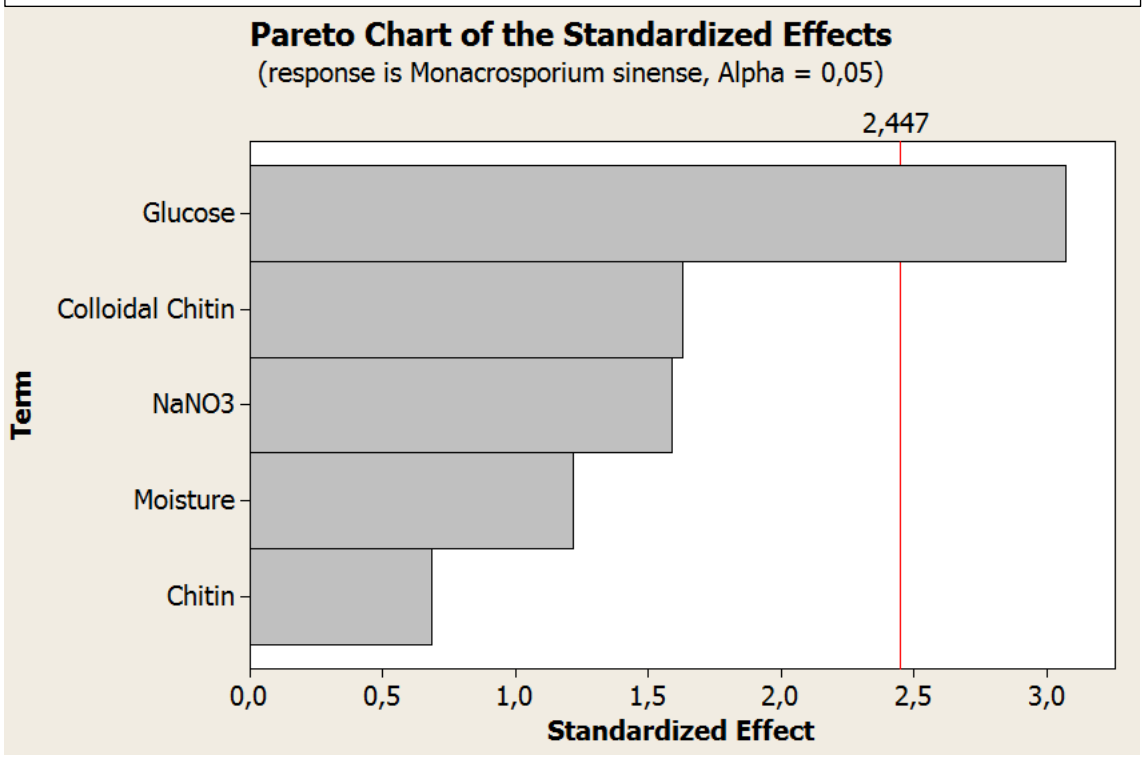


Fig. 1 A–B – Pareto Charts of the studied variables in the Plackett–Burman statistical design.

On the other hand, for SF53, could be seen that, at the levels evaluated, only the variable glucose was significant ($p < 0.05$) (Table 5 and Fig. 1B).

Table 5. Analysis of the factors studied in the Plackett-Burman statistical design of chitinase production by nematophagous fungus *Monacrosporium sinense* (SF53).

	Effect	**P>|*t*|**
Constant	-	0.000
Chitin	-0.0256	0.524
Colloidal chitin	-0.0616	0.155
Glucose	-0.1165	0.022
$NaNO_3$	-0.0598	0.165
Mositure	0.0462	0.267

The results of the influence of pH on chitinase activity of fungi are shown in Figure 2. For *M. thaumasium* the pH value with higher activity obtained was 5.5. However, for *M. sinense*, the highest activity was observed at pH 8.

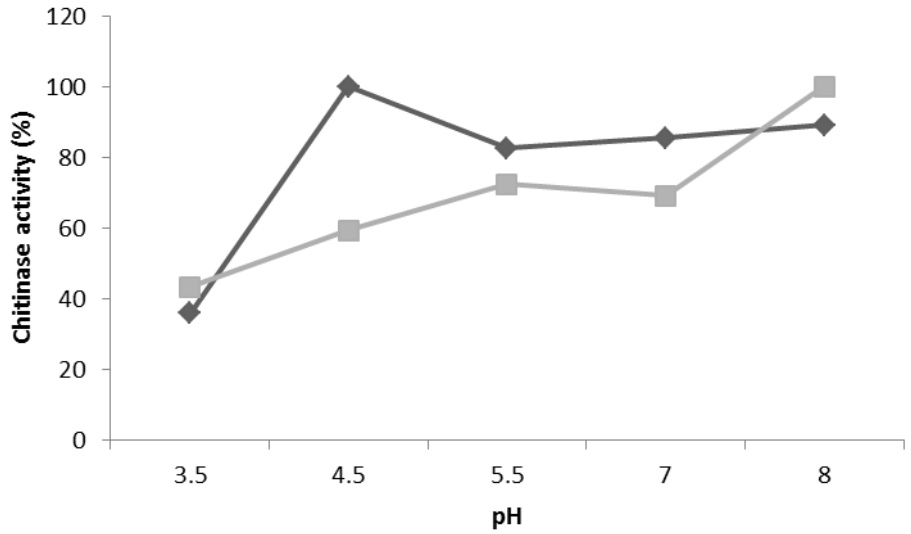


Figure 2. Effect of pH on chitinase activity of the nematophagous fungi *Monacrosporium thaumasium* (NF34) (black) and *M. sinense* (SF53) (gray).

Discussion

In the present study, it was demonstrated that both nematophagous fungi *M. thaumasium* and *M. sinense* have produced chitinase when grown in rich chitin-based medium. To analyze the production of chitinases from nematophagous fungi *M. thaumasium* (NF34) and *M. sinense* (SF53), the Plackett-Burmann factorial design was performed in order to find out which culture variables were significant. It was observed that chitin and $NaNO_3$ are significant for chitinase production by isolate NF34. However, the effect of these variables on the production of chitinase is negative, that is, the lower the level the higher the production of enzyme. Thus, the optimization of the production of chitinase using response surface methodology was not a step to be performed, since the presence of variables negatively affect enzyme production. Moreover, it was observed that the variable glucose was significant for the production of chitinase by the isolate SF53. However, the effect of the variable were also negative, again, the lower the level the higher the production of enzyme. Also in this case, there

was no need for the use of Response Surface Methodology, once the presence of variable negatively affects enzyme production.

Soares et al. (2013a) used the Plackett-Burman statistical design to optimize the production of protease by *M. thaumasium* (NF34). In that study, the authors reported that the nitrogen source was the significant variable on enzyme production, a fact that is consistent with the results of this study, since the nitrogen source ($NaNO_3$) was also significant for the production of chitinase by *M. thaumasium*.

The evaluation of the ability to produce extracellular enzymes that are important in the process infection of nematophagous fungi *versus* nematodes has shown promising results under *in vitro* conditions (Araújo et al., 2004; Soares et al., 2010; Soares et al., 2012). Soares et al. (2012; 2013a,b) reported that fungi from *Monacrosporium* genus are capable of producing protease with nematicidal activity. However, to date, there are no reports of chitinase production by *M. thaumasium* and *M. sinense*, and this is the first report. Thus, the challenge today is to carry out further studies to generate knowledge about the large-scale production of these enzymes from culture media optimized for the growth of nematophagous fungi.

In the present study, the highest chitinase activity was observed at pH 5.5 for *M. thaumasium* and pH 8.0 for *M. sinense*. These data demonstrate that different species of nematophagous fungi may present better enzymatic activity and consequently better predatory activities at different environmental conditions. Thus, further studies are required so that applicability of nematophagous fungi comes to be feasible and scientifically supported.

Conflict of interests

The authors declaree that there is no conflict of interests regarding the publication of this paper.

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CAPÍTULO 6

Nematicidal action of chitinases produced by the fungi *Monacrosporium thaumasium* under laboratorial conditions

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Nematicidal action of chitinases produced by the fungi *Monacrosporium thaumasium* under laboratorial conditions

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Abstract: Nematophagous fungi produce chitinases that may be important in the process of infection of eggs and larvae of nematodes. This study aimed to produce, purify, characterize and test the nematicidal action of extracellular chitinases produced by *Monacrosporium thaumasium* on *Panagrellus redivivus.* Mycelia from *M. thaumasium* were used to inoculate a solid medium for chitinase production. The enzymes were purified using a specific technique of adsorption for chitinases. The chitinase activity was determined at different pHs and temperatures. NF34 produced two distinct chitinases (27 and 30 kDa). After 72 hours, these enzymes provided a significant reduction (80%) ($p < 0.01$) of the number of *P. redivivus* larvae, compared to control. It was shown that isolate NF34 produced chitinases with nematicidal activity. Thus, other experimental designs on geohelminths or even arthropods that transmit diseases may become a new

aspect of the field of study of biological control using predatory nematophagous fungi.

1. Introduction

Chitinases (glycosyl hydrolases that hydrolyze β-1,4-glycosidic bonds between N-acetyl-D-glucosamine and chitin residues) have been detected in a variety of organisms including nematophagous fungi (Gortari & Hours, 2008). The ovicidal and predatory nematophagous fungi produce extracellular enzymes that may be important in the infection process of pests (Mendoza-de Gives et al., 1998; Khan, Williams & Nevalainen, 2004; Braga et al., 2013). In this sense, species within *Monacrosporium* affect nematodes and produce extracellular enzymes which makes them promising candidates for biological control (Araújo, Assis, Campos & Mota, 2004; Soares et al., 2012; Soares et al., 2013). Regarding their caracteristics, conidiophores are branched near their ends and each end carries a conidium, in addition, they produce chlamydospores, which resistence structures (Saxena & Mittal, 1995; Zhang, Yu, Xu & Zhang, 2011).

Knowledge of the constitution of nematode cuticle is important, especially during fungal infection of nematode parasites (Bird & Self, 1995; Araújo, Assis, Campos & Mota, 2004). Cox et al. (1989) report that the protein composition of the parasitic nematode cuticle varies according to the different stages of larval development, which will also influence in the capture of the target nematode (Gronvold, 1996). Several studies have used free-living nematodes of the genus *Panagrellus*, as experimental models mainly due to their availability (Braga et al., 2012). Furthermore, it has been shown that these

organisms can be used to demonstrate the activity of extracellular enzymes produced by nematophagous fungi (Soares et al., 2013).

It is increasingly evident that chitinases are involved in the molecular mechanism of penetration of the cuticle and cellular digestion of nematodes (Huang, Zhao & Zhang, 2004). In this context, Braga et al. (2013) have suggested that nematophagous fungi produce other important enzymes, such as chitinases, in the nematode and arthropod infection process. They have demonstrated that the predatory nematophagous fungus *Duddingtonia flagrans* produces a chitinase which acts on the exoskeleton of ticks. However, there are no reports on chitinase production of an isolate within the genus *Monacrosporium*. Thus, this study aims to produce, purify, characterize and test the nematicidal action of extracellular chitinases produced by the nematophagous fungi *Monacrosporium thaumasium* on the nematode *Panagrellus redivivus.*

2. Material and methods

2.1. Fungus and organism of infection

The free-living nematode *Panagrellus redivivus* has been kept at the Laboratory of Parasitology, Department of Veterinary Medicine of the Federal University of Viçosa, Viçosa, Minas Gerais, Brazil according to Araújo et al. (2004).

Monacrosporium thaumasium (NF34) was obtained from the Fungal Culture Collection of the Laboratory of Parasitology, Veterinary Department, Federal University of Viçosa, Viçosa, Minas Gerais, Brazil. It was maintained in

test tubes at 4°C containing 2% corn-meal-agar (2% CMA) in the dark for 10 days, according to the methodology described by Braga et al. (2009).

2.2. Chitinase production

The chitinase extraction was performed according to Soares et al. (2010). Fungal mycelia were obtained by transferring culture disks (approximately 5 mm in diameter) in 2% CMA to flasks containing solid medium culture. Wheat bran (5 g) supplemented with 1% chitin was used as substrate for chitinase production. Moisture was adjusted to 50% (v/w) using liquid minimal medium. Each flask was covered with hydrophobic cotton, autoclaved at 121°C for 15 minutes and inoculated with a conidial suspension previously prepared and incubated at 28°C for five days (Braga et al., 2013). The enzymes were extracted, centrifuged for 10 min at 10 g at 4°C and the clear supernatant was used for assays (Soares et al., 2010).

2.3. Enzymatic activity

Chitinase activity was measured according to Braga et al. (2013). The colloidal chitin was produced according to the method described by Rodriguez-Kabana et al. (1983). One unit of chitinase is defined as the amount of enzyme required to release 1.0 µmol of N-acetylglucosamine per minute under the assay conditions.

2.4. Purification process and characterization of chitinase

Chitin (0.1 g) was mixed with 1 ml of the clear supernatant obtained from the enzyme extraction in a flask and incubated at 100 rpm at 15°C for 30

min. The mixture was then centrifuged at 13,000 g for 10 min at 4°C. The supernatant was discarded, and the chitin was collected. After the adsorption step, the collected chitin was mixed with 600 µL of Tris-HCl 50 mM pH 8.0 in a flask and incubated at 100 rpm at 15°C for 30 min. Finally, the mixture was centrifuged at 13,000 g for 10 min at 4°C (Braga et al., 2013). The purified proteins were used in subsequent analyses.

Protein content was measured using the method of Bradford (1976). The purification was monitored by SDS-PAGE (Laemmli, 1970). Silver staining was used for the development of protein bands. The molecular weights of the enzymes were estimated by comparison with a standard molecular weight.

Chitinase activity was determined at different pHs using the following buffers: 50 mM sodium acetate (pH 3.5, 4.5 and 5.5) and 50 mM Tris-HCl (pH 7.0 and 8.0). The test temperature was 50°C. Then chitinase activity was determined at different temperature values, at pHs where the highest activities were observed in the previous test: 40, 50, 60 and 70ºC.

2.5. Applicability - Nematicidal assay

Two groups were formed in microtubes, one treated group, where about 50 larvae of *P. redivivus* were incubated together with the chitinases and a control group where about 50 larvae of *P. redivivus* were incubated without the presence of enzymes. Six replicates were performed for each group. Both groups were incubated at 28ºC for 48 hours. After this period, the total number of larvae was counted (Soares et al., 2013). The data underwent analysis of variance at significance levels of 1 and 5% probability. The efficiency of the destruction of the larvae compared to control was assessed by Tukey test at 1%

probability (Ayres, Ayres, Ayres & Santos, 2003). The average percentage reduction of larvae was calculated according to the following equation:

$$\% = \frac{(\bar{x} \text{ of larvae recovered from control} - \bar{x} \text{ of larvae recovered from treatment})}{\bar{x} \text{ of larvae recovered from control}} \times 100$$

3. Results

The isolate NF34 produced two distinct chitinases. However, the methodology used in this study did not allow that each of the enzymes were purified separately (Table 1).

Table 1- Purification process of the chitinases of *Monacrosporium thaumasium* (NF34).

Purification Steps	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	1.62	160.9	0.010	100	1
Adsorption	0.79	23	0.034	32.6	3.43

Therefore, subsequent analyzes were carried out with the two enzymes. The molecular weight of chitinases was estimated at approximately: 27 and 30 kDa (Fig.1).

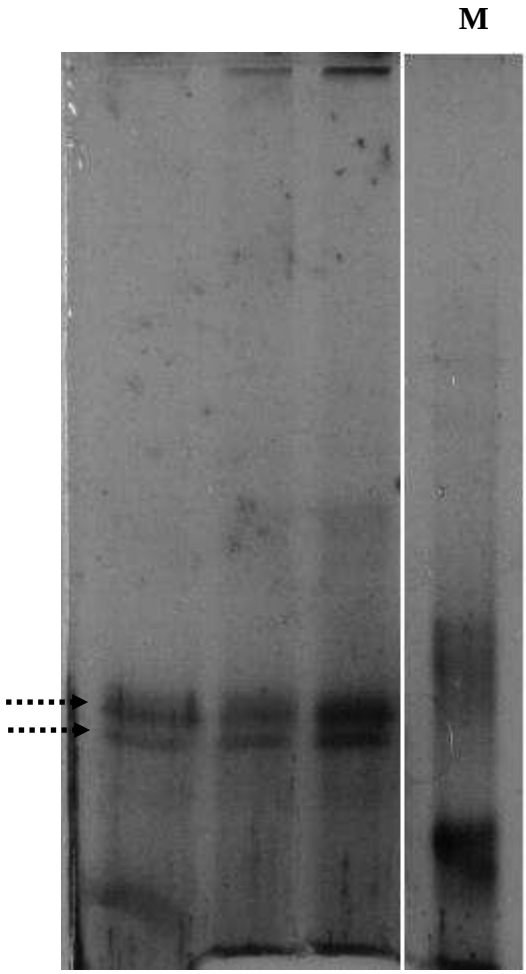

Fig 1. Purification analysis of the chitinases produced by *Monacrosporium thaumasium* (NF34). Line M: molecular weight marker (kDa); Arrows: Purified chitinases.

The enzymatic characterization at different pHs showed two peaks of chitinase activity at pH 4.5 and pH 9 (Fig.2a). Therefore, it is evident that one chitinase has higher activity at acidic pH and the other at basic pH. Thus, the enzymatic characterization was performed at different temperatures in the two pHs of higher activity for each chitinase (pH 4.5 and 9) (Fig.2b).

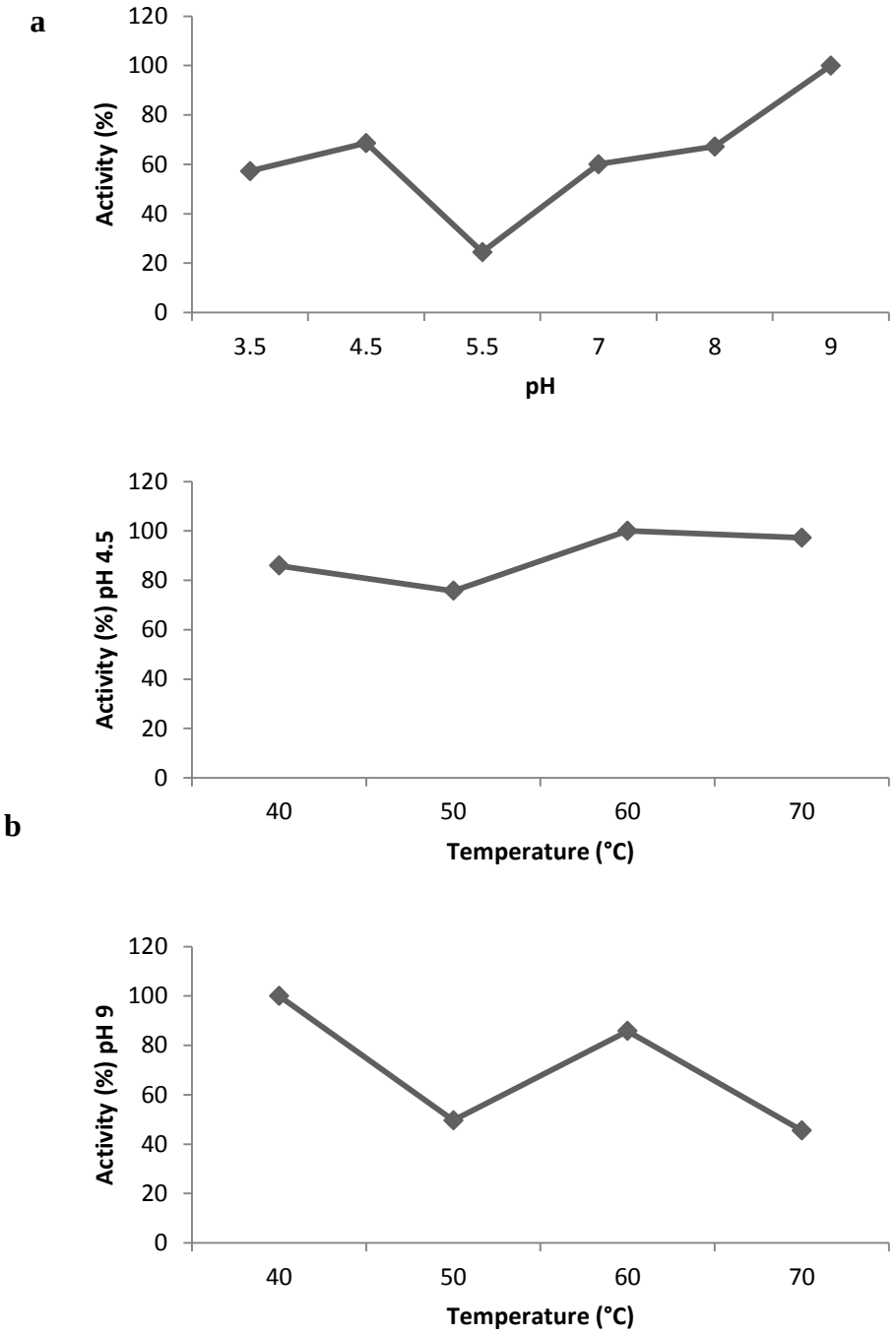

Fig 2. a. E Effect of pH on activity (%) of *Monacrosporium thaumasium* (NF34) chitinases, using the following buffers: 50 mM sodium acetate (pH 3.5, 4.5 and 5.5) and 50 mM Tris-HCl (pH 7.0 and 8.0). The test temperature was 50 °C. **b.** Effect of temperature on activity (%) of *M. thaumasium* (NF34) chitinases. Different temperature values (40 °C, 50 °C and 60 °C) were used at pHs of maximum activity obtained in the previous assay (4.5 and 9).

At pH 4.5 the higher chitinase activity was observed at 60 ºC. On the other hand, at pH 9 the higher chitinase activity was observed at 40 °C. However, as each was not individually analyzed, in the present work we cannot state which of the enzymes present in the gel has one or another feature.

Regarding the nematicidal activity of NF34 chitinases, these enzymes have provided significant reduction of the number of *P. redivivus* larvae, compared to control. These reductions increased with longer incubation times, as can be seen in Table 2 The percentage reductions were 46.5% ($p<0.05$) for 24 hours, 57% ($p<0.01$) for 48 hours and 80% ($p <0.01$) for 72 hours of incubation.

4. Discussion

Species within *Panagrellus* are important biological models used in controlled trials with nematophagous fungi (Braga et al., 2012; Soares et al., 2013). However, this is the first study that demonstrated the activity of chitinases on these organisms, which may, in the future, represent a new step in the understanding of the interaction of extracellular enzymes with the cuticle of the nematode.

The first chitinase (CHI43) of nematophagous fungi with nematicidal activity was described by Tikhonov et al. (2002). In this sense, Braga et al. (2013) have demonstrated the chitinase production by a predatory fungus isolate (AC001). They proposed an assay on engorged female ticks of *Amblyomma cajenense* and highlighted its importance in controlling this arthropod that transmits spotted fever in humans.

Studies have shown that other nematophagous fungi within *Monacrosporium* have been effective in controlling nematodes (Braga et al.,

2009; Soares et al., 2012; Soares et al., 2013; Vilela et al., 2012). However, new approaches have been studied in order to better understand the fungi-*versus*-nematode interaction. The ability of nematophagous fungi in the capture and destruction of pathogens of human and animal health has made this group of organisms a tool which is increasingly exploited in the scientific community. Among these skills, the capture and destruction of nematodes are already consolidated (Gutiérrez et al., 2011; Dias et al., 2013; Paula et al., 2013; Arias et al., 2013). Araújo et al. (2004) report that there is a need to test different isolates of nematophagous fungi according to their characteristics, in particular here, enzyme production.

M. thaumasium enzymatic production and its mechanism of interaction with target organisms still require further studies (Soares et al., 2012). In relation to this fungus, its protease Mt1 showed efficiency in the destruction of *Angiostrongylus vasorum* L_1, a nematode of dogs. A reduction of 23.9% was observed, which demonstrated its importance in the infection process. Moreover, they noted that other proteins, possibly enzymes, also had a role in this process. The chitinases produced by NF34, reduced *P. redivivus* larvae by 46.5% compared to control after 24 hours of incubation. This is interesting, since most studies using nematophagous fungi in Petri dishes have demonstrated averages of reduction of more than 85% (Mendoza de Gives et al., 2006). However, an explanation for the lower percentage of destruction by the enzymes is due to the fact that they were not concentrated, since the objective of this study was to prove their nematicide action and not to concentrate them for a total destruction of nematodes.

This is the first study that demonstrates the activity of *M. thaumasium* (NF34) chitinases on *P. redivivus*, which may, in the future, represent a new step in the understanding of the interaction of extracellular enzymes with the cuticle of the nematode. It was shown that the isolate NF34 produces two distinct chitinases (27 and 30 kDa) with nematicidal activity. Thus, other experimental designs on geohelminths or even arthropods that transmit diseases may become a new aspect of the field of study of biological control using predatory nematophagous fungi.

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4. CONCLUSÕES GERAIS

- Os resultados mostraram que os fungos testados (*Monacrosporium thaumasium* (NF34), *M. sinense* (SF53) e *Pochonia chlamydosporia* (VC4)) produziram enzimas extracelulares com atividade nematicida sobre diferentes nematoides;

- Os estudos das condições de cultura demonstraram que o pH e o tempo de incubação apresentaram efeito significativo para a produção de protease, enquanto os níveis de glicose, quitina e nitrato de sódio influenciaram negativamente a produção de quitinase;

- Três proteases (Ms1, Ms2 e Ms3) e duas quitinases produzidas pelo fungo nematófago *Monacrosporium thaumasium* (NF34) foram identificadas;

- Enzimas extracelulares produzidas pelos fungos nematófagos possuem atividade nematicida e assim mostram um grande potencial para serem utilizados no combate de nematoides que tantos prejuízos causam à agropecuária e também podem infectar os seres humanos;

- Mais estudos devem ser conduzidos no sentido de otimizar as condições de produção de enzima e aplicação destas no combate de nematoides.