UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

RELAÇÃO PATÓGENO-HOSPEDEIRO: ANÁLISE BIOQUÍMICA E PROTEÔMICA DA INTERAÇÃO DO FUNGO *Metarhizium anisopliae* E SEUS HOSPEDEIROS ARTRÓPODES

TESE DE DOUTORADO

LUCÉLIA SANTI

PORTO ALEGRE, ABRIL DE 2009.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

RELAÇÃO PATÓGENO-HOSPEDEIRO: ANÁLISE BIOQUÍMICA E PROTEÔMICA DA INTERAÇÃO DO FUNGO *Metarhizium anisopliae* E SEUS HOSPEDEIROS ARTRÓPODES

LUCÉLIA SANTI

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do grau de Doutor.

Orientadora: profa. Dra. Marilene Henning Vainstein

Co-orientador: prof. Dr. Augusto Schrank

Porto Alegre, abril de 2009.

Este trabalho foi desenvolvido no Laboratório de Fungos de Importância Médica e Biotecnológica, no Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul.

AGRADECIMENTOS

A minha orientadora Prof[a]. Dr[a]. Marilene Henning Vainstein pela confiança durante estes 10 anos, orientação e apoio.

Ao meu co-orientador Prof. Dr. Augusto Schrank pelo apoio.

Às professoras da minha comissão de acompanhamento Dra. Célia Carlini e Dra. Irene Schrank por acompanhar a trajetória do doutorado.

Aos professores da banca pela avaliação do meu trabalho.

Ao Prof. Dr. Alexandre Macedo pela leitura da tese e sugestões.

Aos amigos Renata, Lenise, Ana Paula, Markus, José, Antônio, Lucas, Broetto, Sílvia e Luciano Saucedo, pelas boas gargalhadas, contribuições e sugestões fundamentais.

À Fernanda e ao Gabriel, alunos de iniciação científica, pela amizade e convivência.

Aos colegas de laboratório, especialmente a Simone, Ângela, Melissa, Bianca e Cláudia pelo carinho.

Ao CNPq e CAPES pelo financiamento deste trabalho.

À família Beys da Silva, pelo carinho e por agora ser a minha família também.

Ao meu pai João, onde ele estiver, minha mãe Edeni, meu irmão Diego e minha cunhada Luciana, pela dedicação, amizade, orgulho, confiança e amor.

Ao Walter, meu marido e eterno amor, por ter sido fundamental neste trabalho e por tudo o que representa na minha vida.

ÍNDICE

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

2DE	eletroforese bidimensional
°C	graus celsius
μg	Micrograma
μL	Microlitro
μM	Micromolar
cm	Centímetro
EDTA	ácido etilinodiaminotetracético, sal sódico
g	grama, aceleração da gravidade
h	Hora
kDa	quilodaltons = 1.000 daltons
M	Molar
Mg	Miligrama
Min	minuto
mL	Mililitro
mM	Milimolar
MUF	Metilulbeliferil
nm	Nanômetro
OD	densidade óptica em absorbância em comprimento de onda
pNA	p-nitroanilida
pNP	p-nitrofenil
PAGE	eletroforese em gel de poliacrilamida
pH	potencial hidrogeniônico
Rpm	rotações por minuto
s	Segundos
SDS	dodecilsulfato de sódio
Tris	2-amino-2-hidroximetilpropano-1,3-diol
Triton X-100	éter octilfenólico de decaetilenoglicol
Tween	Polisorbitano

LISTA DE FIGURAS E TABELAS

RESUMO

O fungo filamentoso *Metarhizium anisopliae* é um patógeno capaz de infectar uma grande variedade de artrópodes. A identificação de proteínas e atividades enzimáticas que participem ativamente do processo de infecção é um importante alvo de estudo. Com o objetivo de identificar tais proteínas, uma nova estratégia foi utilizada: imunoproteômica. Estudos relacionados à produção de esporos, formulação e infectividade de *M. anisopliae* para o controle de *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) foram também realizados. Formulações contendo 10% de óleo de soja adicionado a 10^8 conídio.mL^{-1} foi a mais efetiva para ninfas e adultos, sendo as ninfas mais sensíveis ao fungo. Analisando as proteínas extraídas da superfície do esporo, foram identificadas atividades relacionadas à proteção, nutrição e patogenicidade do fungo, como proteases, quitinases, lipases, fosfolipase C (identificada pela primeira vez em esporos), trealase e enzimas envolvidas na proteção contra espécies reativas de oxigênio. Utilizando a metodologia de imunoproteômica diferencial, foram observadas diferenças na secreção de proteínas de *M. anisopliae* em relação aos dois hospedeiros testados: *D. peruvianus* e *Rhipicephalus microplus* (Acari: Ixodidae). Foram identificadas proteases, quitinases e proteínas relacionadas com o processo de infecção em outros organismos (DNase e proteína rica em prolina). Os resultados obtidos neste trabalho indicam que *M. anisopliae* é eficiente no controle de ninfas e adultos de *D. peruvianus*, e formulações contendo 10% de óleo de soja são as mais eficientes dentre as testadas. Um extenso arsenal enzimático foi detectado no sobrenadante de esporos lavados, favorecendo a adaptação do fungo a diferentes substratos, hospedeiros, condições ambientais e nichos de atuação, seja como patógeno ou saprófito. Os resultados de imunoproteômica reforçam a potencialidade do fungo em secretar diferentes proteínas para adaptar-se, no caso, à infecção de *D. peruvianus* ou *R. microplus*, além de evidenciar proteínas e atividades compartilhadas entre os diferentes hospedeiros. As proteínas e enzimas identificadas neste trabalho poderão, isoladamente, ser alvo de novas pesquisas a fim de elucidar o processo de infecção de *M. anisopliae*, em especial à fase inicial da patogênese.

ABSTRACT

The filamentous fungus *Metarhizium anisopliae* is a pathogen that infects a variety of arthropods. The identification of proteins and enzymatic activities that participate actively in the infection process is an important target of study. In order to identify these proteins, a new strategy was used: immunoproteomics. Studies related to the spore production, formulation and *M. anisopliae* infectivity for the control of *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) were also made. Formulations containing 10% of soybean oil added to 10^8 conidia.mL^{-1} was the most effective for nymphs and adults, and the nymphs were more susceptible to fungus. Analyzing the proteins extracted from the spore surface, the activities were identified related to the protection, nutrition and pathogenicity of the fungus, such as proteases, chitinases, lipases, phospholipase C (first identified in spores), trealase and enzymes involved in protection to reactive oxygen species. Using the differential immunoproteomics, differences were observed in the *M. anisopliae* secretion proteins in the two hosts tested: *D. peruvianus* and *Rhipicephalus microplus* (Acari: Ixodidae). Proteases, chitinases and proteins related to the infection process of other organisms (DNase and proline-rich protein) were identified. The results in this work indicate that *M. anisopliae* is effective for the control of *D. peruvianus* nymphs and adults, and that formulations containing 10% of soybean oil were the most efficient between tested. An extensive arsenal was detected in supernatant of washed spores, favoring the adaptation of the fungus to different substrates, host, environmental conditions and niches of action, either as pathogen or saprophyte. The results of immunoproteomics reinforce the capability of the fungus to secrete different proteins to adapt, in this case, the infection of *D. peruvianus* or *R. microplus*, in addition to evidence proteins and activities shared between different hosts. The proteins identified in this work and enzymes may, alone, be the subject of further research to elucidate the infection process of *M. anisopliae*, particularly in the early stages of pathogenesis.

1. INTRODUÇÃO

Pesquisas voltadas para a descoberta de fatores de virulência abrem novas perspectivas para a elucidação da interação patógeno-hospedeiro. Estratégias genômicas ou alternativas para acessar o genoma de organismos (FREIMOSER et al. 2003; DUTRA et al. 2004; WANG et al. 2005; NAGEE et al. 2008; BLUHM et al. 2008), assim como estudos proteômicos e secretômicos (OH et al. 2005; CARBERRY et al. 2006; TSENG et al. 2008) têm permitido a descrição de novos componentes envolvidos em distintos processos biológicos.

Estudos de sequenciamento e proteômica têm auxiliado a descrição de vias metabólicas detalhadas, como a maquinaria envolvida na secreção de proteínas do fungo *Aspergillus niger* (DAVÉ et al. 2006), bem como a descoberta de novos genes ou proteínas em interações patógeno-hospedeiro (FREIMOSER et al. 2005; SUAREZ et al. 2005).

Em *Metarhizium anisopliae*, um dos agentes de controle biológico mais aplicado e bem caracterizado, as interações entre patógeno e hospedeiros estão sendo estudadas (GILLESPIE et al. 1998; FREIMOSER et al. 2005; WANG et al. 2008). O objetivo é conhecer melhor as estratégias utilizadas pelo fungo para infectar seus hospedeiros, além de identificar fatores de especificidade por determinadas classes e/ou espécies de artrópodes.

A estratégia utilizada por *M. anisopliae* para a infecção de seus hospedeiros é a transposição da cutícula de forma direta, através de esporos depositados na superfície do artrópode. Um dos pontos cruciais para o sucesso da infecção é a secreção de enzimas

importantes para a hidrólise dos constituintes da cutícula dos artrópodes, como ceras, proteínas e quitina.

Neste trabalho, o efeito de formulações a base de *M. anisopliae* contendo óleo vegetal sobre ninfas e adultos do percevejo manchador do algodão, *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) foi avaliado, bem como a produção de esporos em arroz. Além disso, visando compreender melhor o processo de infecção, foi utilizada uma nova abordagem para identificar proteínas envolvidas na interação patógeno-hospedeiro: a imunoproteômica, que envolve eletroforese bidimensional seguida por *western blot* e identificação das proteínas. Para analisar o perfil de proteínas possivelmente envolvidas na patogenicidade, foram utilizados dois modelos experimentais: *D. peruvianus* (Insecta) e o carrapato bovino *Rhipicephalus microplus* (Arachnida:Acarina).

2. REVISÃO BIBLIOGRÁFICA

2.1. O controle biológico e *Metarhizium anisopliae*

O controle biológico se baseia em um dos fundamentos básicos das relações ecológicas entre os seres vivos de que cada espécie, seja animal, vegetal ou microbiana, possui inimigos naturais. Pode ser definido como qualquer atividade envolvendo a manipulação de inimigos (predadores, parasitas ou patógenos) naturais ou modificados, e genes ou produtos gênicos para reduzir ou suprir uma população animal ou vegetal que representa uma praga (NATIONAL ACADEMY OF SCIENCE, 1987; MELO & AZEVEDO, 1998). Desta forma, organismos capazes de inibir o crescimento populacional de outros podem ser utilizados no controle de populações específicas que possam vir a se tornar pragas.

Animais, fungos, bactérias e vírus têm papel importante no controle da população de artrópodes e podem ser usados como agentes de controle biológico. Fungos entomopatogênicos podem ser empregados sob três estratégias de controle, denominadas: (i) controle biológico clássico, onde são usados inimigos naturais para controlar uma determinada praga, (ii) aumentação, onde o inimigo natural é aumentado através de inóculos, como um micoinseticida e (iii) conservação, através de modificações do manejo empregado na área afetada a fim de estimular a permanência do fungo (SHAH & PELL, 2003).

O controle biológico é uma alternativa vantajosa em relação ao controle químico, especialmente quanto ao impacto ambiental, custo, especificidade e desenvolvimento de

resistência, não afetando populações de outros artrópodes, como inimigos naturais de pragas ou que são ecologicamente importantes (ALVES, 1998). Em relação aos fungos entomopatogênicos, alguns trabalhos sugerem que os efeitos em artrópodes não hospedeiros são mínimos, tornando-os uma alternativa segura para seu uso em programas de manejo integrado de pragas quando comparados com os inseticidas químicos (GOETTEL & HAJEK, 2000; PELL et al. 2001). A desvantagem encontrada na utilização dos biocontroladores é a necessidade de condições ambientais favoráveis, como temperatura e umidade, para que sejam eficientes. Além disso, o tempo necessário entre a aplicação e a morte dos hospedeiros, em relação ao controle químico, muitas vezes é maior. Esforços têm sido realizados para melhorar a produção em massa, a estabilidade, a formulação e a aplicação de inóculos destes fungos (JENKINS, 1998; GRIMM, 2001; BATTA, 2003; POLAR et al. 2005). Além disso, o entendimento do processo de infecção é fundamental, a fim de acelerar a velocidade de morte do hospedeiro através de estratégias biotecnológicas, como a introdução de genes específicos, tanto do próprio fungo com promotores constitutivos (ST LEGER et al. 1996c; SCREEN et al. 2001), quanto de outros organismos, como uma neurotoxina de escorpião (WANG & ST LEGER, 2007c).

Dentre os microrganismos utilizados para o controle biológico, os fungos entomopatogênicos são os mais utilizados, destacando-se alguns gêneros: *Metarhizium, Beauveria, Aschersonia, Aspergillus, Hirsutella, Nomuraea, Paecilomyces* e *Sporotrix* (SHAH & PELL, 2003). Mais de 700 espécies de fungos pertencentes a aproximadamente 90 gêneros são agentes etiológicos de 80% das doenças de insetos

(ALVES, 1998). Muitos fungos entomopatogênicos sincronizam seu ciclo de vida com o dos hospedeiros (SHAH & PELL, 2003).

Muitos fungos entomopatogênicos são capazes de infectar ativamente a cutícula dos hospedeiros, aliando pressão mecânica, através de uma hifa modificada – apressório, e degradação enzimática. A maioria dos fungos filamentosos não é patogênica para artrópodes por ser incapaz de penetrar sua cutícula. Isto porque (i) não conseguem transpor defesas do hospedeiro na cutícula, (ii) não reconhecem sinais para germinação, formação de apressório e penetração, (iii) não têm enzimas necessárias para a degradação da cutícula, ou (iv) não são capazes de gerar a pressão mecânica necessária para penetrar a cutícula (GILLESPIE et al. 1998).

O fungo filamentoso *Metarhizium anisopliae*, um dos agentes de controle biológico mais aplicado em todo o mundo, começou a ser conhecido no final do século XIX, quando foi testado pelo russo Ilya Metschinkoff para o controle do cartucho de cereais, *Anisoplia austríaca,* e de larvas de *Cleonus punctiventris*, gorgulho praga de beterraba (CLARKSON & CHARNLEY, 1996). Atualmente, os fungos filamentosos estão sendo usados para o controle de pragas em escala moderada em países como China, Austrália, Rússia e Brasil (MILNER, 2000). Além disso, diversos países da Europa e América do Norte utilizam bioinseticidas à base de isolados de *Verticillium lecanii* e espécies de *Metarhizium* e *Beauveria* (CLARKSON & CHARNLEY, 1996).

O gênero *Metarhizium* é composto por três espécies divididas em dez variedades: *M. anisopliae* variedades *anisopliae, majus, lepidiotum* e *acridum; M. flavoviride* variedades tipo E, *flavoviride, minus, novazealandicum* e *pemphigum*; e *M. album*

(DRIVER et al. 2000). Recentemente, uma nova variedade de *M. anisopliae* foi descrita na China: *dcjhyium* (DONG et al. 2007).

Morfologicamente, *M. anisopliae* apresenta micélio hialino e septado, com conidióforos característicos, dos quais emergem conídios cilíndricos organizados em colunas (Figura 1). O desenvolvimento vegetativo desta espécie ocorre normalmente na faixa de temperatura entre 15 e 32 °C, sendo a temperatura ideal entre 24 e 30 °C e pH ótimo de 6,9 (DRIVER et al. 2000; ARRUDA, 2005). No entanto, existe tolerância a um intervalo de pH relativamente amplo (2,0 a 8,5). Os requisitos nutricionais do fungo são poucos, podendo utilizar como fonte de carbono amido, glicose, glicerol, maltose, sacarose e quitina (ALVES, 1998). *M. anisopliae* foi classificado durante muito tempo como Deuteromiceto (TULLOCH, 1976). Entretanto, após o isolamento de *Cordyceps brittlebankisoides*, o teleomorfo de *M. anisopliae* var. *majus*, se propõe sua inclusão em Ascomiceto (LIU et al. 2001).

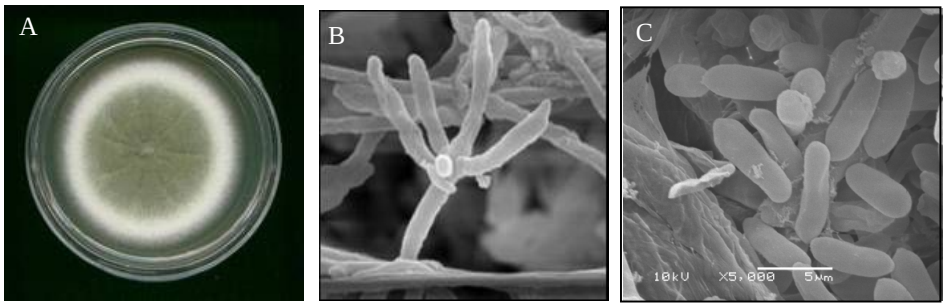


Figura 1- Características morfológicas de *Metarhizium anisopliae* var. *anisopliae*. (A) Cultivo em meio BDA de uma colônia esporulada, mostrando esporos de cor esverdeada (ARRUDA, 2005); **(B)** Microscopia eletrônica de varredura (MEV) mostrando um conidióforo (ARRUDA, 2005); **(C)** MEV de conídios.

M. anisopliae é um entomopatógeno com ampla variedade de hospedeiros, incluindo pragas importantes para saúde publica, da agricultura e da pecuária, sendo capaz de infectar mais de 300 espécies de artrópodes, divididos em 50 famílias (FREIMOSER et al. 2003; ROBERTS & ST LEGER, 2004), além de estar adaptado à rizosfera (HU & ST LEGER, 2002) e ao crescimento em raízes de plantas (ST LEGER, 2008). A Tabela 1 exemplifica alguns hospedeiros para *M. anisopliae*.

Tabela 1- Artrópodes-alvo controlados por *Metarhizium anisopliae*.

Artrópode	Setor	Referência
Aedes aegypti (Vetor da dengue)	Saúde pública	SCHOLTE et al. 2007
Anopheles gambiae (Vetor da malária)	Saúde pública	SCHOLTE et al. 2005
Triatoma infestans (Vetor da doença de Chagas)	Saúde pública	JUAREZ et al. 2000; LAZZARINI et al. 2006
Blatella germanica	Saúde pública	QUESADA-MORAGA et al. 2004
Rhipicephalus microplus	Pecuária	FRAZZON et al. 2000
Loxoceles sp.	Saúde pública	SILVA, 2009- comunicação pessoal
Glossina sp. (Vetor da doença do sono)	Saúde pública	KAAYA & MUNYINI, 1995
Peregrinus maidis	Agricultura	TOLEDO et al. 2007
Dysdercus peruvianus	Agricultura	LUBECK et al. 2008
Capnodis tenebrionis	Agricultura	MARANINNO et al. 2006

No Brasil, um dos países que mais utiliza o controle biológico, *M. anisopliae* é empregado para o controle de pragas da cana-de-açúcar (*Mahanarva posticata*, *M. fibriolata* e *Heterotermes* sp.), das pastagens (*Deois flavopicta*, *Zulia entreriana* e *Cornitermes cumulans*), da broca da bananeira (*Cosmopolites sordidus*) e da broca-dos-citrus (*Diploschma rotundicolle*) (ALVES, 1998). Alguns inseticidas biológicos à base de *M. anisopliae* são comercializados no Brasil: Metanat®, Metabiol®, Biotec® e Metarril®, sendo utilizados principalmente na cana-de-açúcar e pastagens (ALVES, 1998; ITAFORTE, 2009). Em outros países, a comercialização se dá pelos produtos BioBlast® (USA) e MetaGuard® (Índia) para proteção da madeira e controle de cupins, BioGreen e Green Guard (Austrália) para controle de besouros, Destruxin® (Colômbia) para controle de diversos insetos, Bio Stop® (Brasil) para o controle de formigas e Green Muscle® (África do Sul) para controle de gafanhotos (CABI BIOSCIENCE, 1999; MILNER, 2000; LOMER et al. 2001; BIO CONTROLE, 2009; LAVERLAM, 2009). Nas últimas décadas foram desenvolvidos 171 inseticidas/acaricidas, sendo 35,7% destes formulados com *M. anisopliae* (FARIA & WRAIGHT, 2007), indicando a importância de estudos relacionados ao controle biológico, incluindo produção e estabilidade de esporos e formulações.

A utilização de *M. anisopliae* como bioinseticida requer um bom sistema para a produção de esporos. Uma técnica comum em países como Brasil e Nicarágua, é o uso de grãos de arroz como substrato sólido (GRIMM, 2001). Outros substratos podem ser utilizados, como batata, farinha de trigo e grãos de milho. Segundo RANGEL et al. (2004), a produção de esporos de *M. anisopliae* em arroz melhora a resistência à radiação ultravioleta, importante para aplicação a campo.

Para aumentar a permanência do agente de biocontrole, uma boa formulação é considerada fundamental para o sucesso da utilização de fungos entomopatogênicos em larga escala (ALVES & FILHO, 1998). Óleos vegetais podem ser utilizados como componentes em formulações, devido a sua capacidade de proteger os esporos de radiações ultravioleta (MOORE et al. 1992; ALVES, 1998) e aumentar a infectividade de fungos sobre artrópodes, quando comparada com formulações aquosas (KAAYA & HASSAN, 2000).

2.1.1. O inseto manchador do algodão *Dysdercus peruvianus*

Representantes do gênero *Dysdercus* (Hemiptera: Pyrrhocoridae) são fitófagos e parasitam principalmente plantas da família Malvaceae. Apresentam quatro fases de ninfa até chegar à fase adulta.

O percevejo manchador do algodão, *Dysdercus peruvianus* (Guérin Menéville, 1831) (Figura 2), foi encontrado atacando algodão em diversos países da América, África, Ásia e Austrália (GONÇALVES, 2000). As fêmeas de *D. peruvianus*, após fecundação, depositam, em média, 246 ovos em ninhos feitos no chão (GONÇALVES, 2000). A duração do ciclo varia de 25,1 a 68,5 dias conforme a temperatura (MILANO et al. 1999). Com a adoção de técnicas de manejo de pragas no algodoeiro (*Gossypium* sp.), reduzindo a aplicação de agroquímicos, *D. peruvianus* passou a ocorrer com frequência, causando prejuízos consideráveis (MILANO et al. 1999). Tais prejuízos relacionam-se à perda de peso da semente e redução do teor de óleo, além dos danos indiretos, pela

inoculação de microrganismos e, principalmente, por mancharem as fibras do algodão, o que lhes deu o nome vulgar de percevejos manchadores (BRISOLLA et al. 1992).

Atualmente, o método utilizado para controlar esta praga é baseado no uso de inseticidas organoclorados e organofosforados. Porém, os efeitos ambientais negativos dos pesticidas têm levado ao estudo e desenvolvimento de estratégias alternativas.

Recentemente, foi demonstrado o uso potencial de proteínas do tipo canatoxinas (variantes de ureases de *Canavalia ensiformis*) e peptídeos derivados (STANISÇUASKI et al. 2005). Entretanto, adultos de *D. peruvianus* são insensíveis à dieta contendo altas doses de canatoxina (0,04%). Com isso, estudos baseados em controle biológico surgem como alternativas para o controle deste inseto em diversas fases do desenvolvimento.

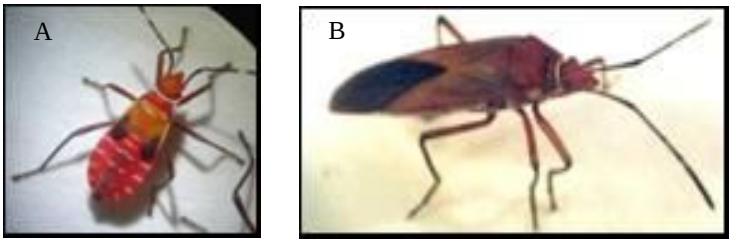


Figura 2 - O percevejo manchador do algodão, *Dysdercus peruvianus*. (A) ninfa de quarto instar; **(B)** adulto.

2.1.2. O carrapato bovino *Rhipicephalus (Boophilus) microplus*

O carrapato *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini) (Figura 3) é um ectoparasito hematófago, cujo principal hospedeiro é o bovino. Habitam áreas tropicais e subtropicais, distribuídos mundialmente entre as latitudes 40°N e 30°S, e estão disseminados por todo o continente americano, exceto Chile e Estados Unidos, onde foram erradicados (FURLONG et al. 2002). Foram introduzidos no Brasil com as primeiras importações de bovinos zebuínos (*Bos indicus*), provenientes do continente asiático, de onde o carrapato é originário (HOOGSTRAAL, 1985). *R. microplus* também afeta o gado híbrido, causando anemia. Assim como outros artrópodes pragas, o controle é feito por acaricidas químicos, o que levou ao surgimento de resistência, principalmente em regiões com alta incidência de carrapatos.

R. microplus acarreta diversos danos econômicos, tornando-se o principal alvo de programas de controle e erradicação nos rebanhos da América do Sul. Um carrapato suga, em média 2 a 3 mL de sangue do hospedeiro, o que reflete em grandes perdas na produção de leite e carne. No processo de alimentação, os carrapatos podem causar ação traumática e espoliativa, pela dilaceração de células e tecidos, bem como ação tóxica, pela inoculação de substâncias através da saliva (LEAL et al. 2003). Além disso, este carrapato pode atuar como vetor de doenças, como a tristeza parasitária bovina, causada por protozoários do gênero *Babesia* e por rikétsias do gênero *Anaplasma* (ANGUS, 1996; LEAL et al. 2003; ESTRADA-PEÑA et al. 2006). Este parasito pode modificar os parâmetros bioquímicos sanguíneos, funções renais e digestivas do hospedeiro (CORREIA et al. 1998; GONZÁLES-ACUÑA & GUGLIELMONE, 2005). GRISI et al.

(2002) relataram que as perdas econômicas causadas por *R. microplus* podem chegar a cerca de dois bilhões de dólares anuais, valores que resultam da diminuição de ganho de peso, gastos com ectoparasiticidas, diminuição da produção de leite, depreciação do couro e lesões contaminadas, predispondo a miíases. No Rio Grande do Sul, a diminuição da produção de carne e da qualidade do couro aumentou devido à introdução do gado da espécie *Bos taurus*, mais suscetível ao carrapato (EVANS et al. 2000).

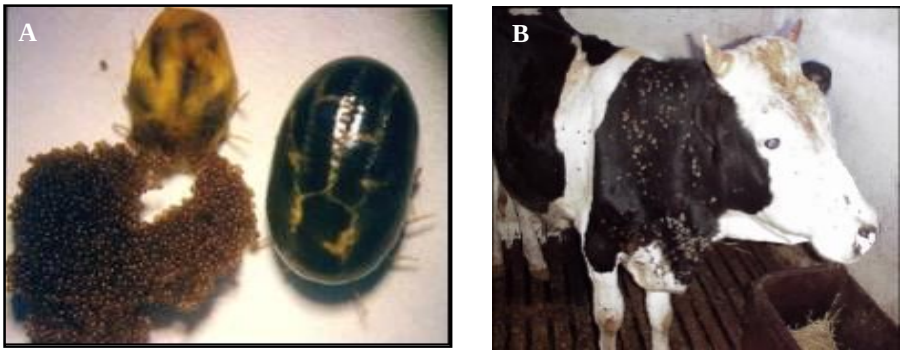


Figura 3 - Teleógina de *R. microplus* em processo de oviposição. (A) Fêmeas de *R. microplus* ovipositando (esquerda), com considerável diminuição de tamanho em relação a fêmea ingurgitada (direita). Tamanho aproximado de 5 mm (http://www.ufrgs.br/depbiot/201). **(B)** bovino com infestação de carrapatos (EMBRAPA Gado de Corte).

2.2. O processo de infecção utilizado por *M. anisopliae*

Esporos de *M. anisopliae* produzidos assexuadamente são responsáveis pela infecção e dispersão no ambiente onde os artrópodes estão presentes. O esporo, em contato com a cutícula do hospedeiro suscetível, fixa-se e germina, iniciando uma cascata de reconhecimento e ativação de reações enzimáticas (SHAH & PELL, 2003).

O processo de infecção inicia com a adesão do esporo mediada por interação hidrofóbica entre proteínas apolares, as hidrofobinas, e a camada lipídica do hospedeiro (ST LEGER et al. 1992; FANG et al. 2007). Estruturas da superfície, topologia e composição química da cutícula dos artrópodes podem afetar a adesão do esporo (LORD & HOWARD, 2004; PEDRINI et al. 2007). *M. anisopliae* é capaz de reconhecer sinais específicos do hospedeiro, provavelmente lipídios da epicutícula, sendo a especificidade decorrente destes sinais (PEDRINI et al. 2007). Segundo ST LEGER et al. (1991a), os componentes presentes na epicutícula são extremamente heterogêneos, inclusive entre artrópodes do mesmo gênero. Recentemente, foi demonstrada a participação de uma adesina (MAD1) na adesão de esporos em larvas de *Manduca sexta*, sendo diminuída a germinação e a virulência em mutantes nulos para o gene que codifica esta proteína (WANG & ST LEGER, 2007a).

Após a adesão dos esporos, a germinação é um passo fundamental para a infecção e, para que isto ocorra, uma fonte de carbono é necessária. FERRON (1985) demonstrou que quitina e certos ácidos graxos foram eficientemente utilizados para este fim, indicando que fungos entomopatogênicos podem utilizar nutrientes presentes na carapaça de artrópodes. Na superfície do esporo ainda não germinado, foi detectada a presença de

enzimas como proteases, esterases e N-acetilglicosidases, que auxiliam na adesão e na aquisição preliminar de nutrientes, causando modificações superficiais na cutícula do hospedeiro (ST LEGER et al. 1990).

Com a adesão e conseqüente germinação do esporo, o sucesso da infecção ocorre pela penetração da hifa, através do desenvolvimento de hifas na superfície do artrópode até a formação do apressório, estrutura especializada para penetração estimulada pelo contato físico com a cutícula (PEDRINI et al. 2007). O apressório acumula açúcares, sendo responsável pela geração de pressão osmótica necessária para a penetração (WANG & ST LEGER, 2007b). Recentemente, foram demonstrados pelo menos quatro sinais diferentes que afetam a formação do apressório: (i) a fração polar da cutícula do hospedeiro; (ii) os níveis de nutrientes; (iii) a superfície hidrofóbica, e (iv) sinais ainda desconhecidos resultantes da carapaça do hospedeiro (WANG & ST LEGER, 2005). Após a formação do apressório, grampos de penetração, caracterizados por uma alteração na parede celular da parte do apressório que está em contato com o hospedeiro, são desenvolvidos (ST LEGER et al. 1991b; CLARKSON & CHARNLEY, 1996).

Evidências obtidas por microscopia eletrônica e histoquímica sugerem que a etapa de penetração ocorre por uma combinação de pressão mecânica e degradação enzimática (ST LEGER et al. 1986a; ARRUDA et al. 2005; SILVA et al. 2005). Neste processo são produzidas enzimas fundamentais para a degradação da cutícula, como lipases/esterases, quitinases e proteases (KUCERA, 1980; ST. LEGER et al. 1986a, 1991b; ALVES, 1998; KRIEGER DE MORAES et al. 2003; SILVA et al. 2005).

Após o processo de penetração, o fungo inicia a etapa de colonização do hospedeiro. As hifas que atravessam a cutícula, sofrem um engrossamento e se

ramificam, liberando toxinas que causam a paralisação do hospedeiro (BIDOCHKA et al. 1997). Estas toxinas, do tipo destruxinas, são peptídeos cíclicos que causam paralisia muscular e inibição da função de hemócitos (células de defesa) e túbulos de Malpighi, além da supressão do sistema imune do hospedeiro (KERSHAW et al. 1999; PAL et al. 2007). A hemocele é rica em nutrientes e açúcares como trealose, oferecendo um ambiente propício para o desenvolvimento do fungo. Nesta fase, a secreção de proteínas é muito diferente em relação à fase de penetração. Ocorre a secreção de proteínas relacionadas à detoxificação, metabolismo de esteróides, estresse e aquisição de nutrientes, como trealases, glicosidases, aspártico-proteases e catalases (WANG et al. 2005; ZHAO et al. 2006, XIA et al. 2002). O tempo de colonização pode variar, dependendo do hospedeiro e das condições ambientais (HSIAO & KO, 2001; WANG et al. 2003).

Após a morte do hospedeiro, as hifas invadem órgãos internos e, com o esgotamento de nutrientes, se estendem para fora do tegumento. Sob condições ambientais apropriadas, ocorre a produção de esporos de coloração verde oliva que podem ser disseminados pelo vento para infectar outros indivíduos (LAVERLAM, 2009). A Figura 4 resume o processo de infecção utilizado por *M. anisopliae*.

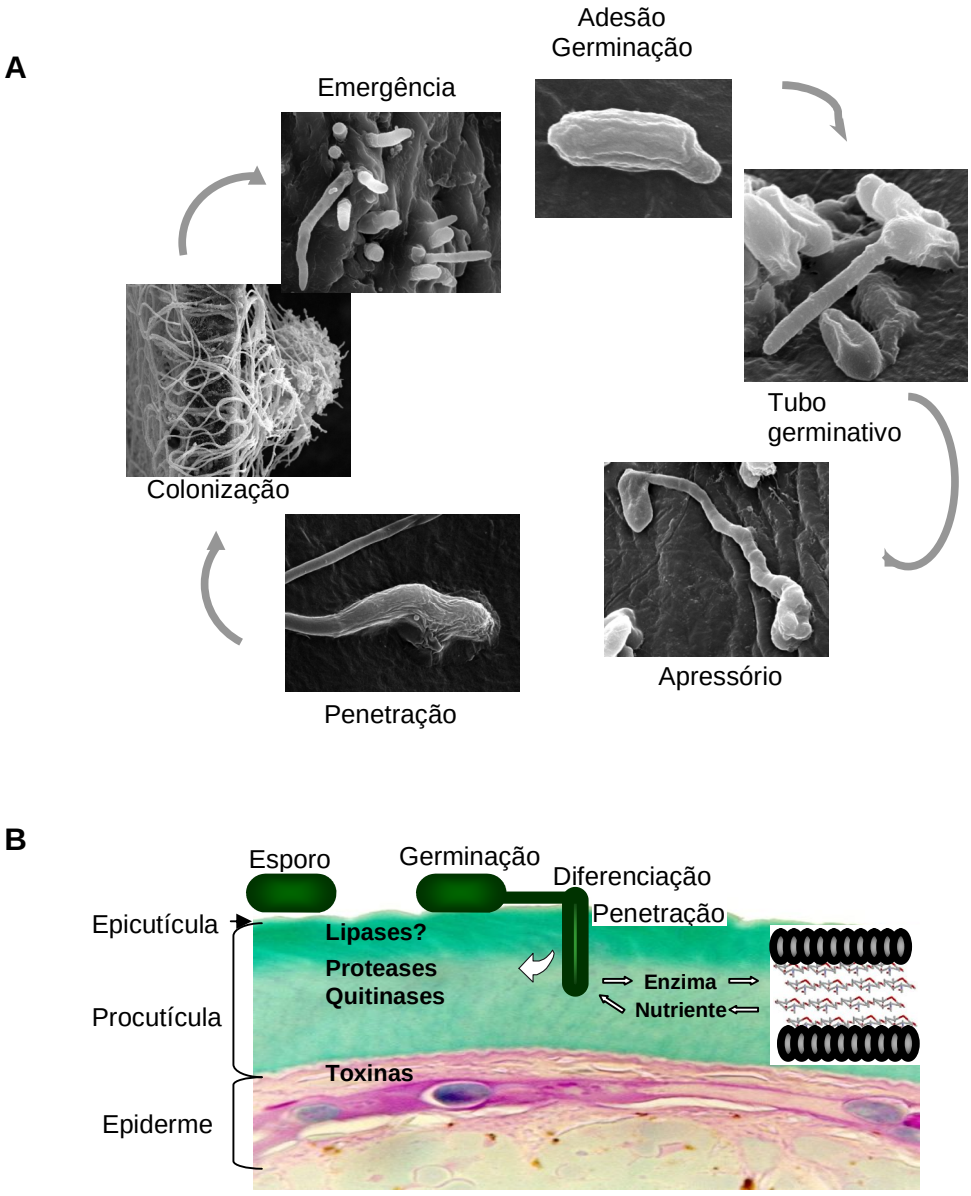


Figura 4 - Ciclo de infecção e possível mecanismo de penetração do fungo *Metarhizium anisopliae* em seus hospedeiros. (A) Ciclo de infecção sobre o carrapato bovino *Rhipicephalus microplus* (modificado de ARRUDA, 2005). **(B)** Durante o processo de penetração na cutícula dos hospedeiros o fungo secreta diversas enzimas hidrolíticas, com função relacionada à aquisição de nutrientes e degradação dos componentes do tegumento do hospedeiro. No detalhe uma representação esquemática da procutícula (cedida por SCHRANK, A.).

A cutícula do hospedeiro é a principal barreira contra infecções e apresenta uma natureza química complexa, sendo formada por duas camadas principais: a epicutícula e a procutícula, seguindo a epiderme e a hemolinfa (Figura 5) (MELO & AZEVEDO, 1998).

A epicutícula é a camada mais externa, muito fina (0,1 - 3 µm), composta por pelo menos duas subcamadas, sendo a mais interna denominada de cuticulina, composta por lipoproteínas, lipídeos polimerizados e polifenóis, e outra mais externa de ceras, formada exclusivamente por lipídeos esterificados a partir de ácidos graxos (em sua maioria de cadeia longa) e álcoois. A função da epicutícula é proteger o inseto contra dessecação, servindo de defesa contra patógenos (BLOMQUIST et al. 1987). Em alguns insetos, pode haver ainda uma camada protetora contra processos abrasivos.

Diferenças na composição dos hidrocarbonetos nas diferentes fases do desenvolvimento dos insetos podem interferir com a suscetibilidade a entomopatógenos e inseticidas, conforme descrito para o besouro *Tribolium castaneum* (AKBAR et al. 2004). Estudos demonstram que alguns ácidos graxos são importantes para a germinação do esporo, indicando que fungos entomopatogênicos podem utilizar nutrientes presentes na epicutícula (BOUCIAS & LATGE, 1988; NAPOLITANO & JUAREZ, 1997).

A procutícula compreende a maior parte da cutícula, sendo sua composição basicamente de natureza protéica e quitinosa (CLARKSON & CHARNLEY, 1996). Nesta porção encontram-se, portanto, sítios específicos para ação de proteases e quitinases.

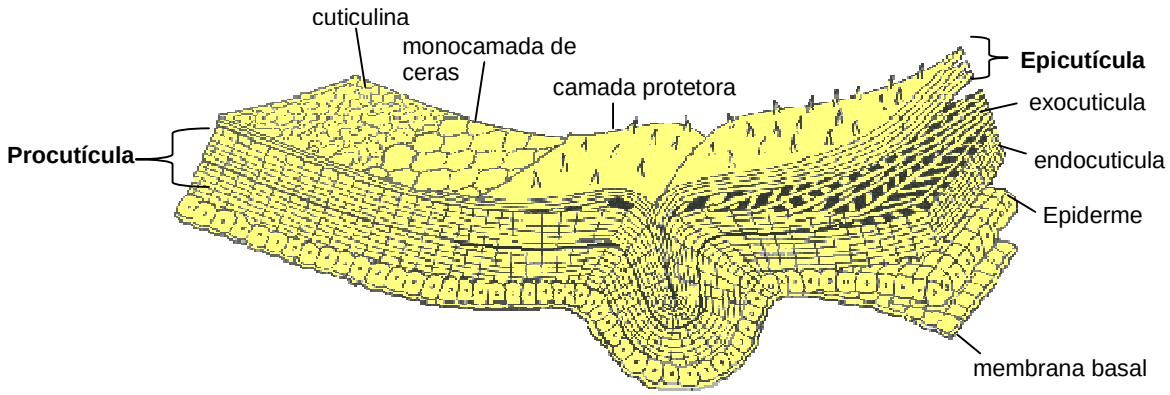


Figura 5 - Constituição básica do tegumento de artrópodes. (SILVA, 2005).

2.3. Possíveis fatores de virulência de *Metarhizium anisopliae*

Segundo SHAPIRO-ILAN et al. (2005), virulência é "o grau de patogenicidade entre um grupo ou espécies". A variação na virulência de linhagens fúngicas sobre uma variedade de artrópodes pode ser relacionada com a produção de enzimas, entre outros fatores (BIDOCHKA & KHACHATOURIANS, 1990).

Muitas enzimas possivelmente envolvidas na virulência de *M. anisopliae* já foram purificadas e sua indução por cutículas de insetos comprovadas (ST LEGER et al. 1986a, PINTO et al. 1997; KRIEGER DE MORAES et al. 2004). Atualmente, outras abordagens vêm sendo feitas a fim de confirmar o papel destas enzimas no processo de infecção e no reconhecimento de hospedeiros, além de identificar outras proteínas participantes destes processos em *M. anisopliae*. Estudos relacionados à superexpressão de genes (ST LEGER et al. 1996c; SCREEN et al. 2001; WANG & ST LEGER, 2006), identificação

de genes diferencialmente expressos em condições de infecção (FREIMOSER et al. 2003, 2005; DUTRA et al. 2004) e proteômica de *M. anisopliae* vêm sendo realizados (MURAD et al. 2008).

Linhagens patogênicas de *M. anisopliae* para uma espécie particular de hospedeiro podem não apresentar as mesmas características de patogenicidade para outras espécies de artrópodes (HUXHAM et al. 1989; LUBECK et al. 2008). Para que as hifas atravessem a cutícula dos artrópodes, o reconhecimento e a resposta do hospedeiro parecem críticos para a seqüência de eventos que culmina em uma infecção eficiente. Sugere-se que várias classes de genes estejam envolvidas nesse processo: (i) aqueles que codificam receptores que detectam a presença do hospedeiro; (ii) aqueles que codificam enzimas que facilitam a penetração; (iii) aqueles cujos produtos inativam as defesas do hospedeiro; e (iv) aqueles que codificam toxinas necessárias para causar sintomas de doença (SCREEN et al. 2001; FREIMOSER et al. 2003).

Em principio, muitas das enzimas hidrolíticas secretadas por *M. anisopliae* são potenciais determinantes de patogenicidade. Entretanto, é necessária que sua participação no processo de penetração e infecção seja experimentalmente confirmada.

As primeiras enzimas comprovadamente secretadas no início da penetração são as proteases, que participam da hidrólise da porção protéica da cutícula. É possível que lipases precedam as proteases devido à camada de composição lipídica que é depositada na epicutícula dos artrópodes (ST LEGER et al. 1986a; SILVA et al. 2005). A atividade quitinolítica decorre à proteolítica devido à estrutura física da cutícula de artrópodes. As fibras de quitina funcionam como sítios de ancoramento para algumas proteínas, estando, portanto recobertas por uma matriz de natureza protéica (BIDOCHKA et al. 1997).

2.3.1. Proteases

As proteases de *M. anisopliae* têm sido alvo de intensos estudos. Durante o processo infectivo do hospedeiro *Manduca sexta*, pelo menos 14 isoformas de proteases com pI entre 4,5 e 10 puderam ser detectadas por zimogramas (ST LEGER et al. 1998). Dentre as proteases produzidas por este fungo, as subtilisinas estão em maior número e destaque, sendo 11 as isoformas encontradas (Pr1A a Pr1K) (BAGGA et al. 2004). As diferentes isoformas das subtilisinas são oriundas de eventos de duplicação gênica e provavelmente estão relacionadas com a patogenicidade, aumento da adaptabilidade em diferentes ambientes e especificidade para o hospedeiro (BAGGA et al. 2004; HU & ST LEGER, 2004).

Quando relacionadas diretamente como fatores de virulência, apenas Pr1A, possivelmente a protease mais importante na degradação da cutícula, tem sido associada à infecção. Quando cópias múltiplas do gene *pr1a* fusionadas a um promotor constitutivo foram inseridas em *M. anisopliae*, a sua eficiência no processo de infecção foi substancialmente aumentada para larvas de *M. sexta* em comparação com a linhagem selvagem (ST. LEGER et al. 1996c). Entretanto, WANG et al. (2002) mostraram que mutantes espontâneos estáveis de *M. anisopliae* deficientes para os genes *pr1a* e *pr1b* são capazes de infectar o inseto *Galleria mellonella* em níveis similares àqueles da linhagem selvagem, mas com uma redução na patogenicidade para outro inseto, *Tenebrio molitor*. Estes fatos demonstram que as proteases não são os únicos fatores envolvidos na patogenicidade (WANG et al. 2002).

Além de proteases do tipo subtilisinas, *M. anisopliae* secreta proteases do tipo tripsina (Pr2) (ST LEGER et al. 1996a; GILLESPIE et al. 1998), quimotripsina (SCREEN & ST LEGER, 2000), cisteíno-proteases (Pr4, que pode ser uma isoforma de Pr2) (COLE et al. 1993), carboxipeptidases (ST. LEGER et al. 1994b; JOSHI & ST LEGER, 1999) e metaloproteases (ST LEGER et al. 1994a). A identificação de inibidores de proteases produzidos pelos artrópodes durante o processo infeccioso constitui um indício da importância desta classe de enzimas para a infecção de *M. anisopliae* em hospedeiros (SASAKI et al. 2008).

Pr1 e Pr2 estão presentes em outros fungos entomopatogênicos, incluindo *V. lecanii, B. bassiana e Nomuraea rileyi* (ST LEGER et al. 1987), implicando a importância funcional para a patogênese. Além disso, Pr2 usualmente é detectada em culturas antes de Pr1, o que sugere o papel de Pr2 na produção de peptídeos indutores para Pr1 (SMITHSON et al. 1995; GILLESPIE et al. 1998). Duas isoformas de Pr2 parecem estar associadas ao apressório, sugerindo que estão disponíveis durante os primeiros estágios da colonização da cutícula (ST LEGER et al. 1996a). Embora o gene que codifica uma destas proteases tenha sido clonado e caracterizado, ainda não está completamente elucidada a função de Pr2 na patogenicidade (SMITHSON et al. 1995).

Recentemente, QAZI & KHACHATOURIANS (2007) cultivaram esporos de *M. anisopliae* durante quatro dias em água (conídios hidratados) e observaram a liberação de pelo menos cinco isoformas de metaloproteases, com massas entre 12 e 103 kDa, sugerindo que a presença destas proteases pode ter significado funcional para o fungo, fornecendo vantagens para a patogenicidade ou saprofitismo.

Nenhuma outra espécie de fungo tem tanta diversidade descrita em proteases como *M. anisopliae*, o que é fundamental para transpor a barreira do inseto e sobreviver em diversos ambientes durante o saprofitismo (FREIMOSER et al. 2003).

2.3.2. Quitinases

As quitinases são outras enzimas hidrolíticas que podem também estar envolvidas na patogenicidade como fatores de virulência para fungos micopatógenos e entomopatógenos (HASSAN & CHARNLEY, 1989; ULHOA & PEBERDY, 1991; DE LA CRUZ et al. 1992; ST LEGER et al. 1998; KRIEGER DE MORAES et al. 2003; SILVA et al. 2005). Provavelmente, estas enzimas atuam sinergisticamente com as enzimas proteolíticas para solubilizar a cutícula do hospedeiro durante a penetração (ST LEGER et al. 1986a, 1987, 1998). Em fungos, além da disponibilização da quitina como nutriente, as quitinases estão envolvidas na modificação da quitina constituinte da parede celular, na liberação de esporos, na diferenciação e na morfogênese de hifas (GOODAY et al. 1992).

Em diversos sistemas fúngicos, as quitinases também desempenham a função de virulência. Em *B. bassiana*, um entomopatógeno conhecido, a superexpressão de uma endoquitinase, denominada Bchit1, ocasionou um aumento da virulência para um afídeo (*Myzus persicae*), diminuindo em 50% a sua concentração letal e em 50% o tempo que levou para matar insetos adultos (FANG et al. 2005). Para o micoparasita *T. harzianum*, linhagens transgênicas contendo cópias múltiplas do gene *ech42* demonstraram aumento na virulência contra o fungo *Rhizoctonia solani*, ao passo em que não houve diferenças

na virulência para o fungo *Sclerotium rolfsii* (CARSOLIO et al. 1999). Outro transformante, superexpessando o gene *chit33*, também apresentou maior virulência contra *R. solani* (LIMÓN et al. 1999).

Para *M. anisopliae*, SCREEN et al. (2001) demonstraram que transformantes superexpressando o gene *chit1* (que codifica uma quitinase de 42 kDa) de *M. anisopliae* var. *acridum* não alteraram a patogenicidade contra *M. sexta*. Porém, isto não significa que estas enzimas não participam da infecção. Recentemente em nosso grupo, SILVA et al. (2005) detectaram pelo menos seis enzimas quitinolíticas em culturas de *M. anisopliae* contendo carapaça de carrapato. Uma destas quitinases, de 30 kDa (CHIT30), apresenta atividade de endo e exoquitinase, tornando-a mais eficaz na degradação da quitina. Foi ainda demonstrada a presença desta quitinase CHIT30 durante o processo de infecção e penetração sobre o carrapato bovino *R. microplus* através de imunolocalização, sugerindo a importância desta enzima no processo de patogenicidade (SILVA et al. 2005). Ainda, STAATS (2007) observou que linhagens mutantes para o gene codificador de CHIT30 apresentavam menor virulência a *D. peruvianus* em relação à linhagem selvagem.

A ação pontual das quitinases em processos específicos como infecção a patógenos requer um mecanismo de indução/repressão, em que a quitina e seus produtos de degradação induzem a secreção de quitinases, enquanto glicose e outras fontes de carbono facilmente metabolizáveis reprimem a secreção (KRIEGER DE MORAES et al. 2003; SILVA et al. 2005). Entretanto, sua regulação não está completamente elucidada, uma vez que transcritos de genes de quitinases aparecem durante o cultivo em meio contendo glicose (BARATTO et al. 2006).

Os fungos filamentosos são capazes de modificar o pH do meio, sugerindo que este fator regula a expressão de genes de virulência de *M. anisopliae*, haja vista as proteases são secretadas em pH básico e as quitinases em pH ácido (ST LEGER et al. 1998).

Assim como para as proteases, ainda não está completamente elucidado o papel das quitinases no processo de entomoparasitismo. Como observado, diversas quitinases são secretadas por *M. anisopliae* em condições de infecção, restando saber quais destas enzimas são importantes para o fungo e para a penetração na cutícula do hospedeiro.

2.3.3. Outros possíveis fatores de virulência

Lipases e esterases estão também supostamente envolvidas no processo de infecção de *M. anisopliae*, pois, dentre os constituintes da cutícula dos hospedeiros, estão os lipídios (ST. LEGER et al. 1986a, 1991a; CLARKSON & CHARNLEY, 1996; SILVA et al. 2005).

Recentemente, nosso grupo comprovou que *M. anisopliae* é capaz de produzir lipases induzidas por diferentes lipídeos como fonte de carbono, sendo o primeiro trabalho a detectar estas enzimas em sobrenadantes de cultura (SILVA et al. 2005). Anteriormente, apenas detecções pontuais de atividade de lipase foram relatadas (ROBERT & AL AIDROOS, 1985; ST LEGER et al. 1986a; NAHAR et al. 2004). Nosso grupo demonstrou, ainda, a presença de lipases durante a infecção de *R. microplus* através de ensaios enzimáticos (SILVA, 2005).

Alguns autores sugerem que a expressão diferenciada das superóxido-dismutases (SODs), enzimas que atuam como um sistema de defesa contra radicais livres de oxigênio (SCHRANK et al. 1993; BITTENCOURT et al, 2004), trealases, enzimas envolvidas na degradação de trealose presente na hemolinfa (XIA et al. 2002) e a presença de micovírus com genoma de dsRNA (GIMÉNEZ-PECCI et al. 2002; TIAGO et al. 2004) podem influenciar na virulência de *M. anisopliae*.

Atualmente, outras abordagens vêm sendo feitas a fim de se identificar proteínas participantes do processo de infecção de *M. anisopliae*, principalmente com o uso de ferramentas moleculares e proteômica, que permitem uma visão global do padrão de expressão e secreção deste fungo frente a componentes estruturais dos seus hospedeiros.

Em experimentos de seqüenciamento de ESTs (*expressed sequence tags*), FREIMOSER et al. (2003) identificaram genes relacionados a patogenicidade de duas subespécies de *M. anisopliae* (var. *anisopliae* e var. *acridum*), incluindo proteases, quitinases, fosfolipases, esterases e fosfatases, importantes para a transposição da barreira cuticular. Outros genes como lacases e monofenol oxigenases, possivelmente envolvidos na mobilização de componentes da cutícula, e catalases e peroxidases, já descritos como envolvidos na patogenicidade de fungos patógenos de animais e plantas, conferindo também proteção contra espécies reativas de oxigênio geradas pelo hospedeiro, foram detectados (WU et al. 1997). Outras ESTs similares a fatores de patogenicidade conhecidos para outros organismos, como fenazina de *Pseudomonas aeruginosa* (MAHJAN et al. 1999) e glicoproteína do tipo mucina, que medeia a invasão do protozoário *Cryptosporidium parvum* (BARNES et al. 1998), também foram identificadas. Em 2005, através da análise da expressão de genes de *M. anisopliae* por

microarranjo cultivado em cutículas de diferentes insetos, foram identificados genes relacionados à degradação de açúcares, metabolismo de aminoácidos e carboidratos, lipases, quitinases, proteases, hidrofobinas e toxinas, entre outros (FREIMOSER et al. 2005). Um fato interessante é que muitos genes regulados positivamente na presença de cutícula de insetos não tinham seu papel na patogenicidade conhecido até o momento, mas que agora podem ser indicados como co-reguladores de fatores de virulência, provendo uma fonte adicional para pesquisas (FREIMOSER et al. 2005). Através da análise de seqüências com expressão diferencial (RDA), DUTRA et al. (2004) identificaram diversos genes regulados positivamente relacionados com metabolismo celular, proteases e resposta a estresse quando *M. anisopliae* foi cultivado em cutícula de *R. microplus.*

2.4. A proteômica

Ao contrário de estratégias moleculares que visam o estudo de genes e analisam o perfil de expressão destes, a proteômica avança no sentido de identificar proteínas secretadas, e se faz de extrema importância no entendimento de diversos processos.

O termo proteômica começou a ser utilizado em 1995 e é definido pela caracterização em larga escala de todas as proteínas expressadas em uma célula, tecido ou organismo (WILKINS et al. 1995). Hoje, este conceito está mais abrangente, uma vez que a proteômica é capaz de quantificar, localizar, determinar modificações pós-traducionais e, principalmente, atribuir função às proteínas identificadas, assim como construir mapas de interações protéicas. Porém, trabalhos relacionados à proteômica

surgiram bem antes, com o sequenciamento de proteínas pelo método de degradação de Edman (EDMAN, 1949) e espectrometria de massas (BIEMANN, 1963).

Diversas metodologias vêm sendo utilizadas para estudos relacionados a proteômica. Para a separação de proteínas, a eletroforese em gel bidimensional (2DE) desenvolvida por O'FARRELL (1975) e otimizada por BJELLQVIST et al. (1982), e a separação por cromatografia líquida (LC) são as mais utilizadas. Embora a técnica de 2DE apresente alguns problemas relacionados com a reprodutibilidade, é capaz de gerar informações importantes a respeito das proteínas, como modificações pós-traducionais, (fosforilação, glicosilação ou proteólise), além da massa molecular e pI (GORG et al. 2007).

A proteômica tem se destacado atualmente como uma ferramenta extremamente importante na descoberta de proteínas envolvidas em diversos processos. Esta técnica é aplicada na elucidação de processos fisiológicos (CARBERRY et al. 2006; KOLKMAN et al. 2006; MATHESIUS, 2008), na localização de proteínas em diferentes tecidos (CAPRIOLI et al. 1997; CHAURAND et al. 2004; MAGALHÃES et al. 2008), na análise de interações proteína-proteína (ARENKOV et al. 2000; JACKMAN et al. 2008), na caracterização de diferentes regiões celulares (ASIF et al. 2006; EUBEL et al. 2008) e relacionadas à interação patógeno-hospedeiro, como micoparasitismo (GRINYER et al. 2005; SUAREZ et al. 2005; TSENG et al. 2008), fitoparasitismo (WANG et al. 2005; COUMANS et al. 2009; SHAH et al. 2009) e entomoparasitismo (MURAD et al. 2006, 2007, 2008).

A imunoproteômica é uma variação da técnica de proteômica, envolvendo *western blot* em géis bidimensionais. Esta metodologia vem sendo aplicada para a

identificação de proteínas imunogênicas, visando o desenvolvimento de vacinas (BARBEY et al. 2008; GENG et al. 2008; WU et al. 2008), testes para diagnóstico (MINI et al. 2006), biomarcadores clínicos (PEDERSEN et al. 2005) e análises comparativas (SHIN et al. 2007). Esta nova abordagem de identificação de proteínas ganha destaque por utilizar anticorpos, tornando a técnica muito mais sensível. Isto favorece o reconhecimento de proteínas pouco concentradas nas amostras a serem analisadas, sem o uso de corantes específicos para sua detecção.

3. OBJETIVOS

Visando uma melhor compreensão da interação de *M. anisopliae* com seus hospedeiros através da identificação de proteínas que possam estar envolvidas neste processo, a imunoproteômica foi utilizada neste trabalho. Além disso, para avaliar proteínas relacionadas com a especificidade patógeno-hospedeiro, dois hospedeiros de diferentes classes de artrópodes foram utilizados: o carrapato bovino *R. microplus* (Arachnida: Acarina) e o percevejo manchador do algodão *D. peruvianus* (Insecta).

Uma vez que o processo de infecção inicia-se com a adesão do esporo, a identificação de diferentes atividades enzimáticas e proteínas presentes em proteínas da superfície do esporo de *M. anisopliae* pode auxiliar no entendimento da relação de patogenicidade e infecção em artrópodes.

Para isto, objetivamos:

(i) estudar o efeito formulações a base de óleo de *M. anisopliae* sobre *D. peruvianus*;

(ii) identificar diferentes atividades enzimáticas em proteínas extraídas da superfície do esporo de *M. anisopliae*;

(iii) detectar e identificar por imunoproteômica proteínas possivelmente envolvidas na infecção;

(iv) identificar proteínas que estejam relacionadas com a especificidade durante o entomoparasitismo em duas diferentes classes de artrópodes (Arachnida e Insecta).

Os resultados desta tese estão organizados em quatro capítulos apresentados na forma de manuscritos científicos.

4. CAPÍTULOS

4.1. Capítulo I - "Effect of the entomopathogenic fungus *Metarhizium anisopliae* and soybean oil formulations on the cotton stainer bug, *Dysdercus peruvianus*"

Manuscrito submetido a Fungal Ecology

Atualmente, o controle de *D. peruvianus* é realizado com inseticidas químicos. Visando a diminuição da contaminação ambiental causada por estes produtos, novas abordagens têm sido estudadas para controlar *D. peruvianus*, como o controle biológico. Este trabalho descreve o efeito de *M. anisopliae* sobre ninfas e adultos do percevejo manchador do algodão *D. peruvianus* utilizando diferentes formulações contendo óleo de soja e Agral, aditivo não iônico adicionado a diversos pesticidas. Como uma alta produtividade de esporos é importante para utilização de *M. anisopliae* como biocontrolador a campo, o uso e reuso de arroz para a produção destes esporos foi também avaliado. Os resultados obtidos indicam que a formulação contendo 10% de óleo de soja adicionado à concentração de 10^8 esporos.mL^{-1} foi a mais efetiva para o controle de ninfas e adultos. Valores de LT_{50} indicam que as ninfas são mais sensíveis ao entomopatógeno, e a análise por microscopia eletrônica de varredura mostra que *M. anisopliae* é capaz de aderir a qualquer parte do corpo do inseto, dando preferência às junções segmentares. Além disso, a reutilização de arroz para a produção de esporos é viável e pode reduzir custos para futuras aplicações a campo.

Dear Dr. Marilene Vainstein,

We have received your article "Effect of the entomopathogenic fungus Metarhizium anisopliae and soybean oil formulations on the cotton stainer bug, Dysdercus peruvianus" for consideration for publication in Fungal Ecology.

Your manuscript will be given a reference number once an editor has been assigned.

To track the status of your paper, please do the following:

1. Go to this URL: http://ees.elsevier.com/funeco/

Thank you for submitting your work to this journal.

Kind regards,

Elsevier Editorial System

Fungal Ecology

Effect of the entomopathogenic fungus *Metarhizium anisopliae* in soybean oil formulations on the cotton stainer bug, *Dysdercus peruvianus*

Lucélia SANTI[a,*], Lucas A. D. SILVA[a,*], Walter O. B. SILVA[a], Ana Paula F. CORRÊA[a], Fernanda STANISÇUASKI[a], Célia R. CARLINI[a,b], Augusto SCHRANK[a], Marilene H. VAINSTEIN[a,#]

[a]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, P.O. Box 15005, 91501-970, Porto Alegre, RS, Brasil

[b]Departamento de Biofísica, Universidade Federal do Rio Grande do Sul, Brasil

[*] Both authors contributed equally for this manuscript.

Short title: *M. anisopliae* oil formulation on *D. peruvianus*

[#] Corresponding author. Tel.: +55 (51) 3308-6060; Fax +55 (51) 3308-7309

E-mail address: mhv@cbiot.ufrgs.br (M.H. Vainstein)

Abstract

The bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae) is an insect pest that causes heavy losses in cotton plantations. The need to reduce the use of insecticides for control of this pest has increased steadily. *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes), well known as an entomopathogenic fungus has thus become a candidate biopesticide. Here, we evaluate the effect of *M. anisopliae* on *D. peruvianus* nymphs and adults using formulations with soybean oil and Agral, and the use and reuse of rice for conidia production. Formulation using 10% soybean oil added to 10^8 conidia mL^{-1} was the most effective for nymph and adult insects, causing 100% of mortality in 6 and 7 days after exposure, respectively. In addition, LT_{50} value indicate that nymphs are more sensitive that adult insects. The SEM analysis of infected insects showed that *M. anisopliae* conidia are capable of attaching anywhere on the body, although preferred attachment sites at junctions. Also, rice reuse is viable and may reduce costs improving commercial conidia production.

Keywords: Biological control, *Dysdercus peruvianus, Metarhizium anisopliae*, Soybean oil formulation, Rice reusing

Introduction

The cotton stainer bug *Dysdercus peruvianus* (Guérin-Menéville) feeds on cotton seeds, causing damage to seeds and stain cotton fibers and leads to heavy economic losses in cotton production. Also, *D. peruvianus* is an important vector of phytopathogenic bacteria and fungi (Gallo *et al.*1988). The life cycle of *D. peruvianus*, from egg o adults, takes 28-32 days at 28 °C, under laboratory conditions, comprising 5 nymphal stages (Milano *et al.* 1999).

The method currently used to control this insect pest is based mainly on the use of organochlorine and organophosphorus insecticides. However, the negative environmental effects of pesticides have brought the need to develop alternative strategies such as biological control. Recently, Stanisçuaski *et al.* (2005) showed the potential use of canatoxin-like proteins and derived peptides for the control of *D. peruvianus*. However, adults were insensitive to diets containing higher concentrations of canatoxin (0.04%). Recent study shows the efficiency of *M. anisopliae* for biological control of fourth instar *D. peruvianus* (Lubeck *et al.* 2008). However, the efficiency of *M. anisopliae* control for adult insects was not evaluated.

The fungus *M. anisopliae* (Metschnikoff) (Liu *et al.* 2001) is a well-characterized entomopathogen. It is studied in control strategies against a diversity of arthropods, including agricultural pests and disease vectors (Luz *et al.* 1998; Wright *et al.* 2004; Scholte *et al.* 2005; Lazzarini *et al.* 2006). In Brazil, it is used to control insect pests in sugarcane plantations, such as *Mahanarva posticata*, and has potential applications against other arthropods, including the bovine tick *Rhipicephalus microplus* (Frazzon *et*

al. 2000; Arruda *et al.* 2005). The mechanisms of infection of some host insects of *M. anisopliae* has been widely studied (Shah & Pell 2003; Roberts & St Leger 2004; Wang & St Leger 2007a; Wang *et al.* 2008).

M. anisopliae utilization for application in biocontrol at field requires a good conidia production system. A usual technique is the growth in solid substrate. In Brazil, several industries utilize rice grain as substrate, to fungus growth. Other substrates have been reported, as potatoes, wheat flour and corn grain.

There are factors which may limit the utilization of microbial insecticides. One of them is the time necessary to insect-pest death (which is longer when compared to conventional pesticides). Therefore, it is necessary to develop correct formulations, i.e. appropriate carriers for host infection. A good formulation is considered fundamental for the successful utilization of entomopathogenic fungus on large scale (Alves & Filho 1998). Vegetable oil may be used as a component in formulation, as a vehicle and for spore, protection from ultraviolet radiation (Moore *et al.* 1992; Inglis *et al.* 1995; Alves *et al.* 1998) and to increase the infectiveness of the fungus over the arthropod when compared to aqueous formulations (Kaaya & Hassan 2000).

In this research, we studied the effect of *M. anisopliae* on *D. peruvianus* nymphs and adults using different oil formulations with soybean oil and Agral wetter/spreader and the rice reuse for conidia production.

Material and methods

Metarhizium anisopliae

M. anisopliae var. *anisopliae* isolate E6 was kept as previously described (Bogo *et al.* 1998). Conidia were collected from fungi grown on complete Cove's medium agar plates (Cove 1966) in 0.01% Tween 80 solution, washed and maintained in sterile water. Prior to use, the conidia concentration was determined by count using a Neubauer hemocytometer and suspensions were diluted in sterile water to concentrations used in this work.

Conidia production

Fungus cultivation for conidia production was performed using 100 g of rice added to a 0.5% peptone solution. The medium cultivation was autoclaved in polypropylene bags for 30 min. In each bag 10^6 spores were inoculated and then cultivated at 28 ^oC for 14 days.

Two methods were used for conidia collection: extraction through sieving and aseptic wash. For conidia sieving, ten bags with grown and sporulated fungus were manually selected and sieved. The conidia obtained were preserved at 4 ^oC and low humidity. The sieved rice was washed (aseptic wash) in 2 L of 0.01% of sterile Tween. The suspended spores were collected and preserved under the same conditions of the material sieved.

For cleaning and reutilization, rice (previously sieved and aseptically washed) was washed with distilled water and manual shaking. After, 130 g of material were weighted, kept in polyropylene bags and sterilized. The cultivation and the procedures to obtain

conidia were repeated under the same conditions previously described. The rice cleaning and reutilization procedure was repeated three times consecutively.

The productivity of the extraction method was determined by counting of colony-forming units (CFU) from resulting conidial suspensions of spore production. Spore suspensions of sieved conidia were performed adding 1 g of sieved material to 50 mL of sterile stilled water. Decimal dilutions of spore suspensions from aseptic wash and sieved conidia were performed and 100 µL were inoculated in Petri plates containing solid Sabouraud medium with a glass spreader. Resulting CFU were counted 72 h after inoculation and incubation at 28 ^{o}C.

Dysdercus peruvianus

Colonies of *D. peruvianus* were maintained in transparent plastic flasks covered with screen tissues. Insects were provided with cotton seeds (*Gossypium hirsutum*) as a food source and sterile water (Stanisçuaski *et al.* 2005).

Insect bioassays and determination of lethal time (LT_{50}) of M. anisopliae oil formulations

Six different formulations were tested in bioassays: 10% soybean oil + 10^8 conidia mL^{-1}; 5% of soybean oil + 10^8 conidia mL^{-1}; 1% of soybean oil + 10^8 conidia mL^{-1}; 10% of soybean oil + 10^7 conidia mL^{-1}; 0.1% Agral (Zeneca Crop Protection, U.K.) + 10^8 conidia mL^{-1} and suspension only with 10^8 conidia mL^{-1}. Formulations containing 10% of

soybean oil or only water without conidia were used as controls. A percentage of 0.05% of Tween 80 was added to all formulations.

Three groups of 20 nymphs (fourth instar) or adults were used in each experiment. Insects of both life stages were externally disinfected with 2.5% hypochlorite solution for 2 s and then washed with sterile saline solution followed by sterile water. The insects tested were totally immersed in a formulation tested for approximately 15 s. After immersion, nymphs and adults were separated into three groups of 20 (60 insects for each treatment) and placed in glass flasks covered with screen tissue and provided with cotton seed and sterile water. All groups were maintained in a humid chamber (> 90% relative humidity), at 28 °C and photoperiod (16L: 8D). Nymphs and adults were observed daily to determine survival and LT_{50} mortality. Three individual experiments were carried out in three replicates.

Scanning electron microscopy (SEM)

For SEM analysis, control and infected insects (from 24 at 96 h) were fixed for 4 days at 4 °C in 2% (v/v) glutaraldehyde, 2% paraformaldehyde with 0.1 M sodium cacodylate buffer at a pH of 7.2. After four days, the specimens were rinsed in buffer, dehydrated in a series of 30 at 100% acetone solutions, dried at critical point in CO_2 (CPD 030 BALTEC), and coated with gold using a sputter-coater (SCD 050 BALTEC). The insects were examined in a Jeol JSM 6060 scanning electron microscope at the Centro de Microscopia Eletrônica da Universidade Federal do Rio Grande do Sul (CME/UFRGS, Porto Alegre/RS).

Statistical analysis

Fifty percent lethal time (LT_{50}) was analyzed using the probit analysis (Raymond 1985). Differences between treatments were compared using the Tukey's mean separation test ($P<0.05$) with software for statistical analysis (SPSS, 2003).

Results

Conidial production

Conidia extracted from cultivated rice for 14 days at 28 ^{o}C were weighted (Table 1). In the first growth, rice was dry, which is ideal for the conidia sieving process. Nevertheless, due to rice washing (for reutilization), humidity was retained, impairing the sieving process. Consequently, the weight of the second growth (102 g) was higher than that of the first (67.89 g). Nevertheless, the CFU counting of the second growth was lower in both extraction methods (Table 1). In the third growth, besides the new drop in productivity, rice was inappropriate for spore sieving due to its paste-like consistency. Fungus sporulation decreased as well, which may be observed in conidia extraction decrease, through medium cultivation aseptic wash (Table 1).

Insect bioassays

One hundred percent of mortality of *D. peruvianus* nymphs and adults was observed 6 and 7 days after exposure to formulation containing 10% soybean oil + 10^8 conidia mL^{-1}, respectively (Fig 1). This formulation was the most efficient, even though it did not significantly differ from the formulation of 5% soybean oil + 10^8 conidia mL^{-1} (Fig 1 and 2). Formulations with 1% soybean oil + 10^8 conidia mL^{-1}, 10% soybean oil + 10^7 conidia mL^{-1} and 10^8 conidia mL^{-1} presented similar performance 7 days after insect exposure, which was not significantly different (Fig 2). Control formulations (only water or 10% of soybean oil without conidia) and 10^8 conidia mL^{-1} + 0.1% Agral had similar effectiveness (Fig 1 and 2).

Table 2 shows that for all formulations tested, nymph died before adult insects. Death of infected nymphs during ecdysis was observed several times, suggesting that nymphs are more susceptible to fungal infection.

SEM analysis

The SEM analysis of infected nymphs and adults showed that *M. anisopliae* conidia are capable of attaching anywhere on the body, although the best attachment sites were observed at junctions in legs and abdomen. Conidia germinated on the host surface and form appressoria (Fig 3C and 3D). Germ tubes were also observed (Fig 3D and 3E). Twenty-four hours after conidia exposure, adherence was observed on the host surface and infection continued with conidia germination and appressoria formation. The hyphae were evident 48 h after spore germination (Fig 3F) and active penetration was observed 48 and 72 h after exposure of conidia. Differences in conidia penetration capacities were

observed with penetration occurring at the joints of legs and lobes. Extensive hyphal growth over the cuticle surface was observed 96 h after exposure conidia. One-hundred twenty hours after exposure, conidia hyphae started to extrude from host cuticle surface (Fig 3I).

Discussion

Entomopathogenic fungi have evolved distinct strategies to attach to insect hosts, varying considerably in their ways of action and virulence (Shah & Peel 2003; Roberts & St. Leger 2004). *M. anisopliae* is widely used for the biological control of agriculture pests and other arthropods (Correia *et al.* 1998; Scholte *et al.* 2005; Lazzarini *et al.* 2006). Here, we evaluate the effect of *M. anisopliae* on *D. peruvianus*, the use of oil formulations and the use and reutilization of rice for fungal growth and sporulation. We have demonstrated that *M. anisopliae* infect and is lethal to the nymph and adult stages of *D. peruvianus*.

As substrate for fungal growth, rice fulfils important conditions for *M. anisopliae* conidia production, considering local viability and cost (Jenkins *et al.* 1998). However, after washing and reutilization, particle agglomeration (rice grains) was observed, which may render fungus growth and sporulation difficult. Substrate reuse may be a strategy to reduce costs, but it impairs production and the sieving process mainly in the third growth. The second growth is viable, as revealed in the results obtained. Besides, *M. anisopliae* spore production on rice grain, as conducted in this work, provided the best results in UV stress studies (Rangel *et al.* 2004), which is fundamental for application in field.

Formulations containing vegetal oils are reported to increase fungus infection in a variety of hosts, when compared to aqueous formulations, including ticks (Kaaya & Hassan 2000; Polar *et al.* 2005), tobacco whitefly, *Bemisia tabaci*, using an emulsion containing coconut/soybean oil (Batta 2003), and for the control of *Schistocerca gregaria*, using cotton oil (Bateman *et al.* 1993). It reacts well with *M. anisopliae* conidia hydrophobicity (Polar *et al.* 2005), has low volatility and is compatible with ultra-low volume techniques (ULV) (Bateman *et al.* 1998). In addition, formulation containing vegetal oils does not require the addition of an adhesive agent. Vegetal oils are non-evaporating components in formulations, even in a low humidity medium. Other characteristic of vegetal oils is that they do not influence conidia germination, and they can be stored at 20 ^{o}C for up to 18 weeks with no loss in conidia viability (Batta 2003). Interaction between formulation and medium must also be considered in bio-insecticides application. Jenkins & Thomas (1996) observed that insects had contact with conidia through residual material from formulation present in vegetation. There may have been a contact between infected and non-infected insects as well, which may characterize horizontal transmission (Bateman *et al.* 1998). One important vegetal oil characteristic is its capacity to protect conidia from solar radiation. Alves *et al.* (1998) demonstrated the efficiency of peanut oil for *M. anisopliae* protection against ultraviolet radiation. The Agral is used for a diversity of pesticides and is recommended for ULV applications (Bateman & Alves 2000). However, the Agral did not strengthen the infectiveness of *M. anisopliae* in *D. peruvianus*.

In the present study, the nymphs were more susceptible to *M. anisopliae* than adults, as evidenced by the LT_{50} values. These results may be possibly explained because

the nymphs are much less sensitive to immunogens, which reflects differences in the sensitivity of the immune system between adults and nymphs (Mullen & Goldsworthy 2006). Also, the epicuticular hydrocarbons had significant differences between nymph or larvae and adult insects, which explain the differences in sensitivity, also reported for *Tribolium castaneum* (Akbar *et al.* 2004). Dead insects infected with *M. anisopliae* developed surface mycosis, but no fungal growth was observed in the control insects, which confirms fungal infection as the cause of death.

M. anisopliae infects both nymphs and adults of *D. peruvianus* by direct penetration of the cuticle, where conidia germinate and differentiate to form an appressoria structure. Infection of *D. peruvianus* by *M. anisopliae* as observed using SEM microscopy is consistent with the described sequence of events that characterizes entomopathogenic fungal interactions (Alves 1998; Deising *et al.* 2000; Arruda *et al.* 2005). The morphological events observed during host infection by *M. anisopliae* are a combination of mechanical pressure and enzymatic degradation (Silva *et al.* 2005), in which conidia adherence to the host surface is in all likelihood mediated by hydrophobic interactions between these surfaces (Bidochka *et al.* 1997; Arruda *et al.* 2005). In *D. peruvianus*, most infection sites occurred at the articulations and lobs, which also have been reported by other researches, showing that *M. anisopliae* conidia are frequently observed on the wings and legs of *Frankliniella occidentalis* (Vestergaard *et al.* 1999).

This study demonstrated that *M. anisopliae* infects both nymphs and adults of *D. peruvianus* and that the use of a specific formulation was shown to be necessary to improve biocontrol process of both life stages of the insect. Also, rice reuse is viable and could reduce costs improving economically commercial conidia production. Further

research addressing the possible synergistic effect of the use of the canatoxin-like proteins or derived peptides (Stanisçuaski *et al.* 2005) and *M. anisopliae* formulation containing 10% soybean oil enhanced its usefulness and attest its efficiency to control the cotton pest *D. peruvianus*.

Acknowledgments

We thank Dr. Giancarlo Pasquali for critical reviews and Laboratório de Proteínas Tóxicas (LABROTOX) for gently handing over the insects for bioassays performances. The work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Akbar W, Lord JC, Howard RW, 2004. Diatomaceous earth increases the efficacy of *Beauveria bassiana* against *Tribolium castaneum* larvae and increases conidia attachment. *Journal of Economic Entomology* **97**: 273–280.

Alves SB, 1998. *Controle microbiano de insetos*. Fundação de Estudos Agrários Luiz de Queiroz, Piracicaba.

Alves LFA, Filho AB, 1998. Formulação de entomopatógenos: uma boa formulação é a base para o sucesso de um inseticida microbiano. *Biotecnologia Ciência e Desenvolvimento* **5**: 32–34.

Alves RT, Bateman RP, Prior C, Leather SR, 1998. Effects of simulated solar radiation on conidial germination of *Metarhizium anisopliae* in different formulations. *Crop Protection* **17**: 675–679.

Alves RT, Bateman RP, Gunn J, Prior C, Leather SR, 2002. Effects of different formulations on viability and medium-term storage of *Metarhizium anisopliae* conidia. *Neotropical Entomology* **31**: 91–99.

Arruda W, Lubeck I, Schrank A, Vainstein MH, 2005. Morphological alterations of *Metarhizium anisopliae* during penetration in *Boophilus microplus* ticks. *Experimental and Applied Acarology* **37**: 231–244.

Batta YA, 2003. Production and testing of novel formulations of the entomopathogenic fungus *Metarhizium anisopliae* (Metschinkoff) Sorokin (Deuteromycotina: Hyphomycetes). *Crop Protection* **22**: 415–422.

Bateman RP, Carey M, Moore D, Prior C, 1993. The enchant infectivity of *Metarhzium flavoviride* in oil formulations to desert locusts at low humidities. *Annals of Applied Biology* **122**: 145–152.

Bateman RP, Douro-Kpindou OK, Kooyman C, Lomer C, Ouambama Z, 1998. Some observations on the dose transfer of mycoinsecticide sprays to desert locusts. *Crop Protection* **17**: 151–158.

Bidochka MJ, St Leger RJ, Roberts DW, 1997. Mechanisms of deuteromycete fungal interactions in grasshoppers and locusts: an overview. *Memoirs of the Entomological Society of Canada* **171**: 231–224.

Bogo MR, Rota CA, Pinto Jr H, Ocampos M, Correa CT, Vainstein MH, Schrank A, 1998. A chitinase encoding gene (chit1 gene) from the entomopathogenic

Metarhizium anisopliae: isolation and characterization of genomic and full-length cDNA. *Current Microbiology* **37**: 221–225.

Correia ACB, Fiorin AC, Monteiro AC, Veríssimo CJ, 1998. Effects of *Metarhizium anisopliae* on tick *Boophilus microplus* (Acari: Ixodidae) in stabled cattle. *Journal of Invertebrate Pathology* **71**: 189–191.

Cove DJ, 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochimica et Biophysica Acta* **113**: 51–56.

Deising HB, Werner S, Wernitz M, 2000. The role of fungal apressoria in plant infection. *Microbes and Infection* **2**: 1631–1641.

Frazzon APG, Vaz junior I, Masuda A, Schrank A, Vainstein MH, 2000. In vitro assesment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus*. *Veterinary Parasitology* **94**: 117–125.

Gallo D, 1988. *Manual de Entomologia Agrícola*. CERES, São Paulo.

Grimm C, 2001. Economic feasibility of small-scale production plant for entomopathogenic fungi in Nicaragua. *Crop Protection* **20**: 623–630.

Inglis GD, Goettel MS, Johnson DL, 1995. Influence of ultraviolet light protectants on persistence of the entomopathogenic fungus *Beauveria bassiana*. *Biological Control* **5**: 581–590.

Jenkins NE, Thomas MB, 1996. Effect of formulation and application method on the efficacy of aerial and submerged conidia of *Metarhizium flavoviride* for locust and grasshopper. *Control Pesticide Science* **46**: 299–306.

Jenkins NE, 1998. Development of mass production technology for aerial conidia for use as mycospesticides. *Biocontrol News and Information* **19**: 21–31.

Kaaya GP, Hassan S, 2000. Entomogenous fungi as promising biopesticides for tick control. *Experimental and Applied Acarology* **24**: 913–926.

Lazzarini GM, Rocha LF, Luz C, 2006. Impact of moisture on in vitro germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma infestans*. *Mycological Research* **110**:485–492.

Liu ZY, Liang ZQ, Whalley AJS, Yao YJ, Liu AY, 2001. *Cordyceps brittlebankisoides*, a new pathogen of grubs and its anamorph, *Metarhizium anisopliae* var. *majus*. *Journal of Invertebrate Pathology* **78**: 178–182.

Lubeck I, Arruda W, Souza BK, Stanisçuaski F, Carlini CR, Schrank A, Vainstein MH, 2008. Evaluation of *Metarhizium anisopliae Rhipicephalus (Boophilus) microplus Dysdercus peruvianus*. *Fungal Ecology* **1**: 78–88.

Luz C, Tigano MS, Silva IG, Cordeiro CM, Aljanabi SM, 1998. Selection of *Beauveria bassiana* and *Metarhizium anisopliae* isolates to control *Triatoma infestans*. *Memórias do Instituto Oswaldo Cruz* **93**: 839–846.

Milano P, Consoli FL, Zerio NG, Parra JRP, 1999. Thermal Requirements of the Cotton stainer *Dysdercus peruvianus* Guérin-Méneville (Hemiptera: Pyrrhocoridae). *Anais da Sociedade Entomologica do Brasil* **28**: 233–238.

Moore DP, Bridge D, Higgins PM, Bateman R, Proir C, 1992. Ultra-violet radiation damage to *Metarhizium flavoviride* conidia and the protection given by vegetable and mineral oils and chemical sunscreens. *Annals and Applied Biology* **122**: 605–616.

Mullen LM, Goldsworthy GJ, 2006. Immune responses of locusts to challenge with the pathogenic fungus *Metarhizium* or high doses of laminarin. *Journal of Insect Physiology* **52**: 389–398.

Polar P, Kairo MT, Moore D, Pegram R, John SA, 2005. Comparison of water, oils and emulsifiable adjuvant oils as formulating agents for *Metarhizium anisopliae* for use in control of *Boophilus microplus*. *Mycopathologia* **160**: 151-157.

Rangel DEN, Braga GU, Flint SD, Anderson AJ, Roberts DW, 2004. Variations in UV-B tolerance and germination speed of *Metarhizium anisopliae* conidia produced on insects and artificial substrates. *Journal of Invertebrate Pathology* **87**: 77–83.

Raymond M, 1985. Presentation d'un programme d'analyse logprobit pour micro-ordinateur. *Cahiers Orstom Entomology Medical and Parasitology* **22**: 117–121.

Roberts DW, St Leger RJ, 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. *Advances and Applied Microbiology* **54**: 1–70.

Scholte E, Ng´habi K, Kihonda J, Takken W, Paaijmans K, Abdulla S, Killeen GF, Knols BGJ, 2005. An entomopathogenic fungus for control of adult African malaria mosquitoes. *Science* **308**: 1641–1652.

Shah PA, Peel JK, 2003. Entomopathogenic fungi as biological control agents. *Applied Microbiology and Biotechnology* **61**: 413–423.

Silva MV, Santi L, Staats CC, Costa AM, Colodel EM, Driemeier D, Vainstein MH, Schrank A, 2005. Cuticle-induced endo/exoacting chitinase CHIT30 from *Metarhizium anisopliae* is encoded by an ortholog of the *chi3* gene. *Research in Microbiology* **156**: 382–392.

SPSS Inc, 2003. *SPSS Statistical Software* CD-ROM version 13.0 for Windows. SPSS Inc, Chicago, IL, USA.

Stanisçuaski F, Ferreira-da Silva CT, Mulinari F, Pires-Alves M, Carlini CR, 2005. Insectical effects of canatoxin on the cotton stainer bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). *Toxicon* **45**: 753–760.

Vestergaard S, Butt TM, Bresciani J, Gillespie TA, Eilenberg J, 1999. Light and electron microscopy studies of the infection of the western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae) by the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* **73**: 25–33.

Wang C, Duan Z, St Leger RJ, 2008. MOS1 osmosensor of *Metarhizium anisopliae* is required for adaptation to infect host hemolymph. *Eukaryotic Cell* **7**: 302–309.

Wang C, St Leger RJ, 2007a. The *Metarhizium anisopliae* perilipin homolog MPL1 regulates lipid metabolism, appressorial turgor pressure, and virulence. *Journal of Biological Chemistry* **282**: 21110-21115.

Wright C, Brooks A, Wall R, 2004. Toxicity of the entomopathogenic fungus, *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) to adult females of the blowfly *Lucilia sericata* (Diptera: Calliphoridae). *Pest Management Science* **60**: 639–644.

Table 1 - Production of *Metarhizium anisopliae* conidia in rice. Weight obtained and CFU were examined for three consecutive conidia production in rice.

Growth	Total weight (g)*	Log from CFU of conidia extraction procedure**	
		Manual sieving	Aseptic wash
First	67.89	9.78^{b}	9.97^{a}
Second	102	8.77^{d}	9.26^{c}
Third	30	7.03^{e}	8.79^{d}

*obtained through manual sieving of ten polypropylene rice bags, as described in Methods

**average of seven plates

^{a,b,c,d,e} means followed same letter are not significantly different according Tuckey's test ($P<0.05$).

Table 2 - Lethal time (LT_{50}) values in days following immersion of *D. peruvianus* nymphs and adults in oil formulations containing *M. anisopliae* conidia (95% confidence).

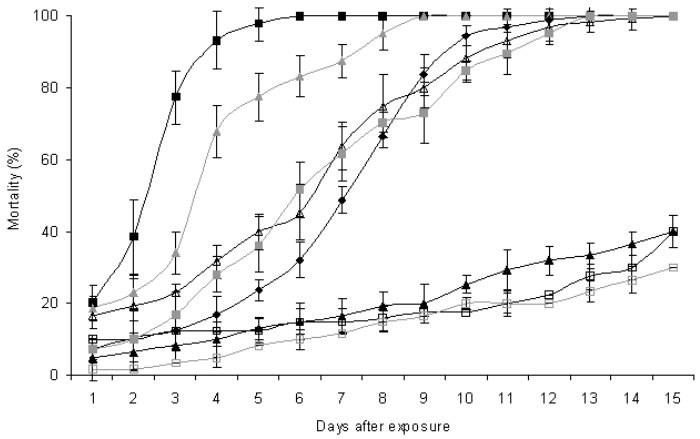
Treatment	Nymphs				Adults			
	LT_{50} (lower – upper)	χ^2	df	*P*	LT_{50} (lower – upper)	χ^2	df	*P*
10% soybean oil + 10^8 conidia mL^{-1}	2.13 (1.89 – 2.36)	6.45	1.3	0.0001	2.46 (2.05 – 2.81)	16.61	1.9	0.0001
5% soybean oil + 10^8 conidia mL^{-1}	3.52 (3.26 – 3.77)	6.5	2.5	0.0001	3.78 (3.46 – 4.09)	9.77	2.5	0.0001
1% soybean oil + 10^8 conidia mL^{-1}	6.52 (6.27 – 6.78)	12.72	4.3	0.0001	6.86 (6.58 – 7.16)	17.73	4.3	0.0001
10% soybean oil + 10^7 conidia mL^{-1}	5.66 (5.37 – 5.95)	11.34	4.3	0.0001	5.97 (5.63 – 6.31)	15.6	4.3	0.0001
0.1% Agral + 10^8 conidia mL^{-1}	22.34 (20.03 – 25.69)	1.94	2.8	0.883	26.58 (23.11 – 32.06)	1.48	2.8	0.98
10^8 conidia mL^{-1}	6.23 (6.07 – 6.4)	4.2	4.3	0.511	6.95 (6.63 – 7.27)	14.31	4.3	0.0001
10% soybean oil (control)	17.21 (15.97 – 18.85)	8.16	4.3	0.0001	17.36 (15.8 – 19.59)	13.37	4.3	0.0001
Water (control)	19.59 (18.44 – 21.02)	4.0	4.3	0.601	18.46 (17.44 – 19.72)	2.53	4.3	0.985

The LT_{50} values were determined using the probit analysis (P = 0.0001).

Legend figures:

Fig 1 - Effect of *M. anisopliae* formulations on *D. peruvianus* (I) nymphs and (II) adults. (■) 10% soybean oil + 10^8 conidia.mL^{-1}; (▲) 5% soybean oil + 10^8 conidia.mL^{-1}; (♦) 1% soybean oil + 10^8 conidia.mL^{-1}; (△) 10% soybean oil + 10^7 conidia.mL^{-1}; (□) 0.1% Agral + 10^8 conidia.mL^{-1}; (▲) 10% soybean oil; (■) water + 10^8 conidia.mL^{-1}; and (□) water (control). Bars represent standard error.

I



II

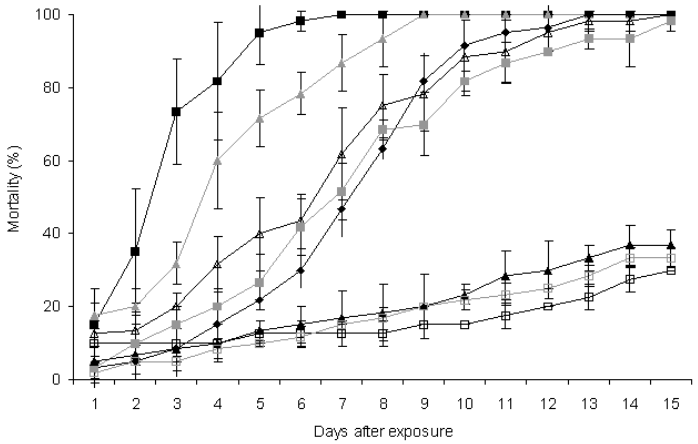
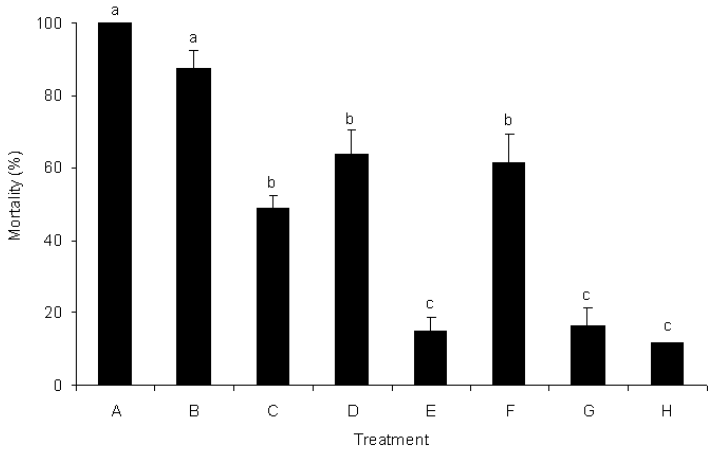


Fig 2 - Mortality of *D. peruvianus* (**I**) nymphs and (**II**) adults with different *M. anisopliae* formulations, 7 days after exposure. (A) 10% soybean oil + 10^8 conidia.mL^{-1}; (B) 5% of soybean oil + 10^8 conidia.mL^{-1}; (C) 1% of soybean oil + 10^8 conidia.mL^{-1}; (D) 10% of soybean oil + 10^7 conidia.mL^{-1}; (E) 0.1% Agral + 10^8 conidia.mL^{-1}; (F) 10^8 conidia.mL^{-1}; (G) 10% soybean oil; and (H) water (control). Means with the same letter are not significantly different according to the Tukey's test (P>0.05). Bars represent standard error.

I



II

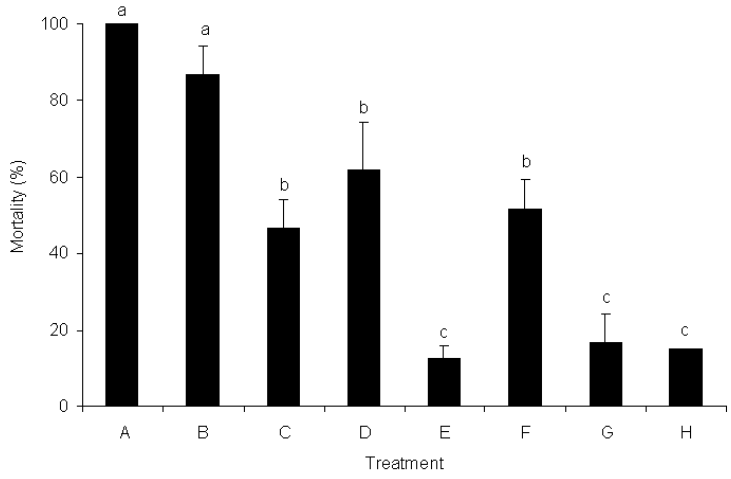
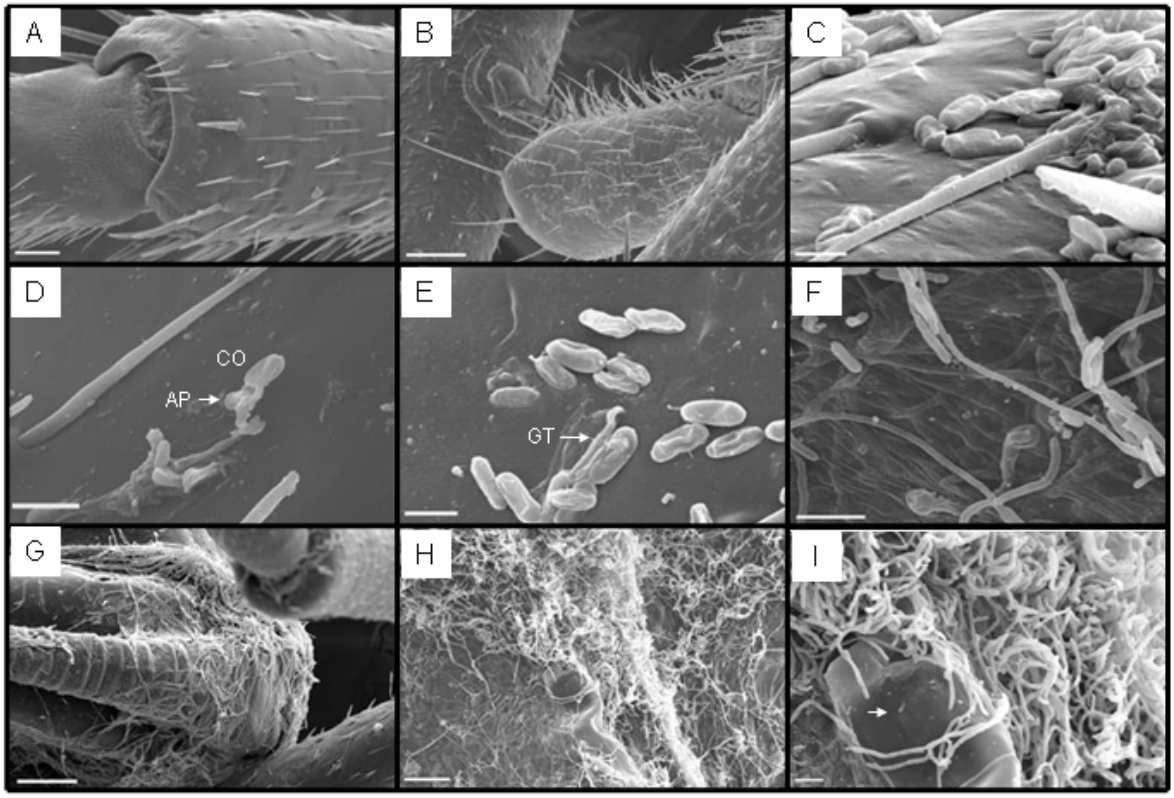


Fig 3 - Scanning electron microscopy (SEM) of *D. peruvianus* exposed to *M. anisopliae* conidia. (**A**) Control (not exposed), bar = 50 μm; (**B-C**) 24 h after conidia exposure, bar = 100 μm and 5 μm; (**D-E**) 48 h after conidia exposure, bar = 10 μm and 5 μm; (**F**) 72 h after conidia exposure, bar = 10 μm; (**G**) 96 h after conidia exposure, bar = 100 μm; (**H-I**) 120 h after conidia exposure (10^8 conidia.mL^{-1}), bar = 50 μm and 10 μm. Details of conidia (CO), germ tube (GT) and appressoria (AP). Arrow in **I** indicates extrusion of hyphae. (**A-B**) legs; (**C-D-E-F-H-I**) abdomen and (**G**) head of *D. peruvianus*.



4.2. Capítulo II – "Conidial surface proteins of *Metarhizium anisopliae*: source of activities related with toxic effects, host penetration and pathogenesis"

Manuscrito submetido a Toxicon

Para o entomoparasitismo, o contato do conídio com a superfície do hospedeiro é o primeiro passo para o sucesso da infecção. Este artigo descreve o perfil enzimático de proteínas da superfície do esporo de *M. anisopliae*. Foram detectadas atividades de proteases (Pr1 e Pr2), quitinases (endo- e exo-quitinases), lipases, esterases, fosfolipase C, trealase e enzimas relacionadas à proteção contra radicais livres. Através de zimogramas, foi observada a presença de isoformas de proteases e quitinases. Estas atividades detectadas no sobrenadante contendo proteínas da superfície do esporo podem auxiliar a compreender os mecanismos envolvidos no contato de *M. anisopliae* com o hospedeiro, tornando o fungo apto a responder rapidamente e favorecer o processo de infecção.

Ms. Ref. No.: TOXCON-D-09-00159

Title: Conidial surface proteins of Metarhizium anisopliae: source of activities related with toxic effects, host penetration and pathogenesis

Toxicon

Dear Dr Marilene Henning Vainstein,

Your submission entitled "Conidial surface proteins of Metarhizium anisopliae: source of activities related with toxic effects, host penetration and pathogenesis" has been assigned the following manuscript number: TOXCON-D-09-00159.

You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/toxcon/.

Thank you for submitting your work to this journal.

Kind regards,

Frankee Woodham-Kay

Journal Manager

Toxicon

Conidial surface proteins of *Metarhizium anisopliae*: Source of activities related with toxic effects, host penetration and pathogenesis

Lucélia Santi[a], Walter O. B. Silva[a], Markus Berger[a], Jorge A. Guimarães[a,b], Augusto Schrank[a,b], Marilene H. Vainstein[a]*

[a] Centro de Biotecnologia Universidade Federal do Rio Grande do Sul, P. O. Box 15005, CEP 91501-970, Porto Alegre, RS, Brazil

[b] Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, P. O. Box 15005, CEP 91501-970, Porto Alegre, RS, Brazil

*Corresponding author: M. Vainstein; Fax +55 (051) 3308-7309, Phone +55 (051) 3308-6060. E-mail: mhv@cbiot.ufrgs.br

Abstract

Conidial contact with an arthropod surface is the first step of the fungal penetration and infection process. However, conidia of *Metarhizium anisopliae* have associated components, like enzymes that could be involved in triggering the penetration process and toxic effects that have not yet been well characterized. Fungi produce many enzymes that also are toxic components found in bacteria and animal venoms and thus may be considered as potential virulence factors. In this work, we report several enzymatic activities from spore surface protein extracts. The major proteolytic activities observed in spore surface proteins (SSP) were Pr1 and Pr2 activities, in that order. According to the zymograms obtained, SSP contain different proteases. SSP contain trehalase, exo- and endo-chitinase activities, and seven different chitinase bands which have been observed in zymograms. Activities involved in protection against reactive oxygen species (ROS) were also detected. Two lipolytic enzymes were also detected in lipase zymograms. Phospholipase C activity, closely related to microbial pathogenesis, was detected for the first time in *M. anisopliae* conidia. These activities described could be an initial step towards understanding the mechanisms involved in the first stage of *M. anisopliae* infection process and its toxic effects against arthropod hosts.

Keywords: Chitinase; Conidia; Host-infection; Lipase; *Metarhizium anisopliae*; Protease; Spore surface protein.

1. Introduction

The entomopathogenic filamentous fungus *Metarhizium anisopliae* is a widespread pathogen of arthropods utilized in biological control strategies against several agriculturally and medically import pests (Frazzon et al., 2000; Shah and Pell, 2003; Quesada-Moraga et al., 2004; Scholte et al., 2005).

During *M. anisopliae* host infection, several enzymatic activities related to penetration, toxic effects, and pathogenicity have been characterized (Charnley, 2003). The participation of proteases, mainly Pr1 proteases, and chitinases in the infection process is evident and has been demonstrated (Freimoser et al., 2005; Silva, MV et al., 2005; St Leger et al., 1996b). Other enzymes already described in this fungus, such as lipase (Silva, WOB et al., 2005), superoxide dismutases (Schrank et al., 1993) and trehalase (Xia et al., 2002) among others, in adition to other unknown enzymes in *M. anisopliae*, could be also related with infection and associated with conidial surface.

The importance of enzymatic activities like lipolytic activity at the initial stages of fungal infection has been widely reported and closely related to infection systems of several pathogens (Davies et al., 2000; Voigt et al., 2005) including enzymes present in the spore surface of filamentous fungus (Berto et al., 1999), which have a crucial role in host infection. Recently, Qazi and Khachatourians (2007) found metalloproteases isoforms in hydrated conidia characterized as metalloproteases, however, neither Pr1 nor Pr2 activities were detected. Besides associated components of conidia, the infective unit

of *M. anisopliae*, which could be utilized to unleash the host penetration and infection process, has not yet been well characterized nor understood.

The aim of this work was to identify, quantify, and characterize different enzymatic activities related to penetration, toxic effects, and pathogenic process in conidial surface of *M. anisopliae*.

2. Material and methods

2.1. Microorganism, conidia production and extraction of spore surface proteins (SSP)

M. anisopliae var. *anisopliae* E6, originally isolated from spittlebug (*Deois flavopicta*) in state of Espírito Santo, Brazil, was kept as previously described (Bogo et al., 1998). Fungus cultivation for conidia production was performed using 100 g of rice added to 30 mL of a 0.5% peptone solution in polypropylene bags. In each sterilized bag, 10^6 spores were inoculated and the fungus was grown at 28 °C for 14 days. Sporulated fungus was mechanically removed from rice grains by gently shaking using a plastic spatula, and then sieved to collect conidia. The spores obtained were suspended in an extraction buffer (Tris HCl 50 mM pH 8.0 containing 0.25% Triton X-100, 1:2.5 w/v) (Silva, WOB et al., 2005). The suspension was vigorously shaken for 5 min and the resulting supernatant was filtered in a 0.2-μm filter (Millipore, USA). Thus obtained the supernatant, called spore surface proteins (SSP), was used as the source for enzymatic assays and zymograms.

2.2. Enzymatic assays

Lipolytic activity was assayed using different ρ-nitrophenyl (ρNP) ester substrates: ρNP-palmitate (ρNPP), ρNP-myristate (ρNPM), ρNP-butirate (ρNPB) and ρNP-acetate (ρNPA) (all from Sigma Chem. Co., St. Louis, USA). The SSP (10 μL) were mixed with 90 μL of substrate solution at pH 8.0 (Silva, WOB et al., 2005). After 30 min of incubation at 37 °C, absorbance was measured at 410 nm against an enzyme-free control using a plate spectrophotometer Spectramax (Molecular Devices, Sunnyvale, USA). The enzymatic activity was calculated with reference to a calibration curve of standard solution of ρ-nitrophenol (Sigma Chem. Co., St. Louis, USA). One lipase unit (U) was defined as the amount of enzyme that releases 1.0 μmol ρ-nitrophenol per hour.

Phospholipase C (PLC) activity assay was evaluated using ρ-nitrophenylphosphorylcholine (ρNPPC), which is a chromogenic substrate specifically cleaved by PLC as described by Kurioka and Matsuda (1976). Next, ρNPPC was used at a concentration of 20 mM in 50 mM Tris–HCl pH 8.0 and 60% sorbitol. Spore surface proteins (10 μL) were mixed with 90 μL of substrate solution and incubated at 37 °C for 1 h. Absorbance was read at 410 nm and one unit of PLC was defined as described above for lipolytic activity unit.

The chitinase activity was assessed with fluorogenic substrates 4-methylumbelliferyl (4-MU) diacetylchitobioside, 4-MU triacetylchitotrioside and 4-MU tetracetylchitotetraoside (Selvaggini et al., 2004). Assays were performed in microtitre plates, with 0.8 mM substrate, 0.1 M McIlvaine buffer pH 5.0 and 20 μL of SSP, in a

final volume of 100 µL per well. Samples were incubated at 37 °C for 30 min. The reaction was stopped by adding 0.1 M glycine/NaOH buffer pH 10.6, and after 5-min incubation, a final reading was obtained. One chitinase unit (U) was defined as the amount of enzyme that releases 1 ρmol of 4-methyllumbelliferone per min, in the assay conditions described above.

For the protease assay, chromogenic substrates were used as previously described (Berger et al., 2008). 10 µL of SSP was incubated in 20 mM Tris–HCl buffer, pH7.4. The reactions were initiated by adding DL-BAPNA (benzoyl-DL-arginine-ρNA) or other synthetic peptide chromogenic substrates as S-2160 (Suc-ala-ala-pro-phe-ρNA), S-2222 (Bz-ile-glu-gly-arg-ρNA), S-2238 (HD-phe-pip-arg-ρNA), S-2251 (HD-val-leu-lys-ρNA), S-2302 (HD-Pro-phe-arg-ρNA), S-2337 (Pip-gly-arg-ρNA) and S-2366 (Pir-glu-pro-arg-ρNA) at 0.2 mM (final concentration), in a final volume of 100 µL. Kinetic assays were monitored at 37 °C for 30 min in a SpectraMax spectrophotometer equipped with thermostat and shaking systems. One protease unit (U) was defined as the amount of enzyme that produces one ρmol of ρ-nitroaniline per hour, in the assay conditions described.

The trehalase activity was assayed using the amount of glucose released from the hydrolysis of trehalose (Xia et al., 2002). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 µg of glucose per hour in the assays conditions described above.

The catalase activity was assayed using hydrogen peroxide as substrate (Cakmak and Marschner, 1992). Phosphate buffer was added along with H_2O_2 10 mM to 25-µL sample aliquots. Catalase activity was estimated by the decrease in absorbance of H_2O_2 at

240 nm for 3 min. The decomposition of H_2O_2 was followed at 240 nm (E = 39.4 mm cm^{-1}).

The assay for superoxide dismutase (SOD) was conducted according to Beyer and Fridovich (1987). A solution containing 0.05 M potassium phosphate buffer pH 7.8, 13 mM L-methionine, 75 mM NBT (nitrobluetetrazolium), 0.1 mM EDTA and 0.025% Triton X-100 was added to glass tubes. To start the reactions, the sample and 10 mM riboflavin were added, at the same time that tubes were placed under fluorescent light for 15 min. After this period, absorbance was determined at 560 nm. SOD unit was defined by NBT reduction per $mL.h^{-1}$.

The peroxidase activity was determined by measuring the absorbance at 470 nm of tetraguaiacol produced by the reaction between 0.05 M guaiacol solution and 10.3 mM hydrogen peroxide solution in 0.1 M phosphate buffer pH 7.0 at 25 °C (Vieira et al., 2003). The increase in absorbance at 470 nm was monitored for 5 min. One activity unit was defined as the amount of enzyme which cause an increase of 0.001 in absorbance per hour under the conditions described above.

Protein was quantified according to the bicinchoninic acid method (BCA), using bovine serum albumin as the standard (Smith et al., 1985).

2.3. Effect of metals and inhibitors on protease activity

To measure inhibitory activity and effect of metals against protease activity, we used Suc-ala-ala-pro-phe-ρNA and Bz-phe-val-arg-ρNA as substrates. The metals $CaCl_2$, $ZnCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $CuCl_2$ and $BaCl_2$ were tested with the final concentration

of 5 mM, and for inhibitors we used PMSF (phenylmethanesulphonylfluoride) with the final concentration of 10 mM, benzamidine 10 mM, $HgCl_2$ 1 mM, EDTA 5 mM and EGTA with the final concentration of 5 mM. For all assays, inhibitors or metals were incubated with the sample for 20 min at room temperature prior to the addition of substrates.

2.4. Zymograms

Gel electrophoresis was carried out with polyacrylamide gels using Mini-Protean III SDS-PAGE system (Bio-Rad).

The detection of the lipase activity in 10% polyacrylamide native gel was performed using the fluorogenic substrate 4-methylumbelliferyl (MUF)-butyrate after electrophoresis (Diaz et al., 1999).

The detection of chitinase activity was performed using copolymerized 0.1% glycol chitin as the substrate. Gels were stained with 0.01% Calcofluor white M2R. After 10 min, the brightener solution was removed and lytic zones, representing digested chitin, were visualized in UV light (St Leger et al., 1993).

For protease detection, electrophoresis was performed in 12% polyacrylamide gel containing 0.2% copolymerized gelatin (Sigma). After electrophoresis, the gels were incubated for 16 h (37 °C) in a renaturation buffer (0.05 M Tris buffer pH 8 containing 2.5% Triton X-100). Protease activity against gelatin was detected by staining residual gelatin with Coomassie blue R-250 (St Leger et al., 1996a).

The catalase activity detection in 7.5% polyacrylamide native gel was performed using 0.01% hydrogen peroxide as substrate after electrophoresis (Zimmermann et al., 2006).

To detect of superoxide dismutase (SOD) activity in gel, proteins were separated on an 8.5% polyacrylamide native gel and stained using NBT, riboflavin and TEMED (Tolfo Bittencourt et al., 2004). Potassium cyanide (KCN) and hydrogen peroxide (H_2O_2) were used at 10 mM to ascertain the SOD type.

3. Results

In this research, we adopted an extraction procedure to remove the surface proteins present in spores of *M. anisopliae*, with the purpose of identifying and analyzing the presence of enzymes, which have physiologic and ecologic relevance, as those associated with the host infection processes. Several enzymatic activities were detected in SSP (Table 1), including proteases, chitinases, lipases, and enzymes involved in stress response. Proteases present in SSP were able to hydrolyze all the different synthetic peptides substrates used. However, we observed certain preference for subtilisin (Pr1) substrate (suc-ala-ala-pro-phe-ρNA) and trypsin (Pr2) substrate (Bz phe-val-arg-ρNA). Protease activity was significantly reduced by Zn^{2+} and Cu^{2+} and completely inhibited by PMSF in both substrates. A significant reduction in activity was reached with Hg^{2+} when Pr2 substrate was used (Table 2). In addition, the metal chelator agents EDTA and EGTA increased activity by 96 and 92% respectively, when Pr1 substrate was used. The zymogram containing gelatin (Fig. 1B) revealed at least five protease isoforms, which varied between 30 and 100 kDa, with the prevalence of high molecular mass proteases.

Chitinase activity was detected with synthetic substrates $4MU_4$ (endochitinase), $4MU_3$ (endo-) and $4MU_2$ (exochitinase) (Table 1), with at least seven activity bands found in the zymogram containing glycol chitin as substrate (Fig. 1B). Trehalase activity (182.9 ± 7.37 U) was also detected in SSP.

Lipolytic enzymes were found in *M. ansiopliae* SSP. Lipase and lipase/sterase activities were detected. Results with different ρNP esters indicate a certain preference for short acyl chain substrates (butyrate and acetate) by the lipolytic enzymes present in spores. The highest activity was 29.24 ± 0.8 U with ρNP-butyrate substrate. Moreover, in native gel, two lipolytic activity bands were detected (Fig. 2B). Phospholipase C activity was also detected in *M. anisopliae* SSP (Table 1).

As for enzymes involved in stress protection, we detected at least one catalase and one SOD in zymograms (Fig. 1B). According to the inhibitory activity for SOD, we identified the activity band as a Cu-Zn SOD in SSP (data not shown). Peroxidase, SOD and catalase activities quantified in *M. anisopliae* were respectively 420 ± 0.32, 302.6 ± 18.95 and 2418 ± 22.21 U (Table 1).

4. Discussion

Entomopathogenic fungi, such as *M. anisopliae* and *Beauveria bassiana* (Fan et al., 2007) produce extracellular enzymes that degrade proteinaceous, lipidic, and chitinous components present in insect cuticle, which allow hyphal penetration through the cuticle to access the nutrient rich insect haemolymph. It has been noted that fungi

produce and secrete many enzyme types that also are toxic components in bacterial and animal venoms, which consequently makes them potential virulence determinants (Screen and St Leger, 2000; Charnley, 2003). The catalytic activities of enzymes produced by fungi are similar to bacterial and animal venoms, which result in harmful consequences to the specific target host. The present study revealed the presence of multiple enzymes of *M. anisopliae* associated with conidia surface, which are considered essential in the arthropod infection process and to the protection against environmental stress (Pedrini et al., 2007; Roberts and St Leger, 2004).

Proteases are an important sub-class of enzymes secreted by *M. anisopliae* that degrade cuticle. The proteolytic activity has usually been characterized using peptides substrates in animal venoms (Pinto et al., 2006). Here, we used a similar approach for identification of *M. anisopliae* entomotoxic activities. Activities of several protease sub-subclasses, EC 3.4_, were reported for *M. anisopliae*: serine proteinase (subtilisin-like Pr1A-K) (Bagga et al., 2004); trypsin-like (Pr2), carboxypeptidase (Joshi and St Leger, 1999), metalloproteases (St Leger et al., 1998), among others. Among the substrates tested, remarkable activity was detected for subtilisin-like proteases (Pr1) and trypsin-like proteases (Pr2). Pr1 activity was approximately 4 times as high as that of Pr2, a finding supported by the fact that Pr1 is recognized as very important in the early stages of the infection process (Small and Bidochka, 2005) and that the decline in spore bound Pr1 was directly correlated with decline in virulence (Shah et al., 2007). Besides, subtilisin-like serine proteases Pr1 form an important family of proteases involved in fungal infection, widely reported for *M. anisopliae* (Bagga et al., 2004; Gillespie et al., 1998) and other entomopathogenic fungi like *Cordyceps sinensis* (Zhang et al., 2008) and

Beauveria bassiana (Donatti et al., 2008). In this study at least five protease isoforms in conidia surface, as revealed in zymograms containing gelatin, were detected,. According to our results, SSP proteases exhibit the capacity to recognize and hydrolyze synthetic substrates with singular amino acid sequences. This protease diversity produces the complex array of chemical weapons that enable fungi to adapt to a broad array of habitats as saprophytes and pathogens (Bagga et al., 2004). We can suggest that multiproteases could complement and digest most proteins present in host cuticle, and that they may offer selective advantage and contribute to the complexity of the host-pathogen interactions, since those multiproteases show different specificities for different substrates.

When the effect of inhibitors and metals on protease activity was tested, we observed a complete inhibition for PMSF, a specific inhibitor of serine proteases such as subtilisin-like Pr1 and trypsin-like Pr2, and a remarkable increase in activity with metal chelators EDTA and EGTA. This is contrarily to results obtained for hydrated conidia (Qazi and Kachatourians, 2007), which identified only metalloproteases. The contact of conidia with host cuticle is the first stage of fungal infection and probably requires a wide diversity of enzymes for host recognition to trigger the infection process. Also, the different metal inhibition or stimulation in protease activity results for both substrates could be associated with regulation of Pr1 and Pr2 activity in different environmental conditions.

The fungus *M. anisopliae* secretes several chitinases related to fungal growth and host cuticle degradation (St Leger et al., 1996b). The regulation of secretion using different substrates has been correlated, including tick cuticles and chitin (Krieger de

Moraes et al., 2003). Conidial surface proteins of *M. anisopliae* contain several chitinases, as visualized in zymograms. The chitinolytic activities observed could be related not only with fungal infection, but mainly with cell wall modifications and triggering of conidia germination. This fact is supported by previous work, which reported chitinolytic activity in the second stage of fungal infection (St Leger et al., 1996b).

Lipolytic enzymes have been described as toxic activity components in venoms (Dani et al., 2005) and as virulence factors in several microbial pathogens with different roles in the infection process (Stehr et al., 2003; Schofield et al., 2005). One of such roles could be the enhancement of the adhesion to the host cuticle, which is experimentally supported by the presence of lipase activity in *M. anisopliae* fungal spores. As described by Göttlich et al. (1995), lypolytic activity increases hydrophobic interactions by releasing free fatty acid, favoring adhesion to the host. In this work, we detected different lypolytic activities in conidial surface that could also be directly linked to fungal virulence. Lipase, with a crucial role in infection of host, has been reported in spores of the phytopatogenic fungus *A. brassicicola* (Berto et al., 1999) and possibly plays a role related to the adhesion and/or penetration of the fungal propagules during the early stages of host-parasite interactions. Moreover, lipids in epicuticle are the first barrier against arthropod pathogenic microorganisms, which reinforces the importance of these enzymes in penetration and initial infection stages, such as the contact of conidia with host surface (first stage).

Phospholipases C (PLC) hydrolyzes the phosphodiester bond in the phospholipid backbone to yield 1,2-diacylglycerol, which depends on the specific phospholipid species

involved. We detected, for the first time, phospolipase C activity in *M. anisopliae* conidia. This activity has been reported to be a virulence factor in several microbial pathogens (Ghannoum, 2000). To aid in the invasion of host tissues, microbial cells possess constitutive and inducible hydrolytic enzymes, such as phospholipases, with toxic implications (Ibrahim et al., 1995), which hydrolyze phospholipids that destroy or derange host cell membrane components, leading to membrane dysfunction and/or physical disruption (Salyers and Witt, 1994).

Trehalose is an important sugar found in spores of many fungi, including *M. anisopliae*, and is the predominant disaccharide in haemolymph of many insects, like *M. sexta* and *Schistocerca gregaria* (Liu et al., 2004). The trehalase activity observed in SSP could be explained because this activity in conidia is a major event during early conidial germination in fungi and presumably provides glucose for energy regulation (Rangel et al., 2008). Besides, this activity could be related with host trehalose consumption to offer nutritional support to the fungus during host colonization.

Enzymes involved in protection against reactive oxygen species (ROS) formed by solar ultraviolet radiation (UV-A and UV-B) and heat are fundamental for conidia protection in the environment. Catalase, Cu-Zn SOD, and peroxidase activities, which are important to spore protection in the environment, were found in SSP. High tolerance to UV radiation is very important to the successful use of *M. anisopliae* as commercial biocontrol agent in crop protection strategies. Also, catalase activity protects against cytotoxic effects of host-derived H_2O_2, as reported for *Claviceps purpurea* in rye (Garre et al., 1998). In addition, *M. anisopliae* spore production on rice grain, as conducted in this work and in commercial production of conidia for biological control, provided the

best results in UV stress studies (Rangel et al., 2004) that could be associated with these enzymatic activities found in SSP.

The diversity of enzymes in *M. anisopliae* spore surface reflects the ability of fungus to respond and attack the host in pathogenesis. This work also detected, for the first time in conidia, an important activity of phospholipase C closely related to microbial pathogenesis and toxic effects. This wide enzymatic arsenal comprises activities related to several physiologic and ecologic functions that are vital for fungal survival and adaptation in a broad array of habitats, such as saprophyte or pathogen. Activities related with conidia protection and germination, adhesion to host, fungal nutrition, cell wall, and membrane cell modification and disruption, which are obviously important to pathogenesis and host infection were identified, characterized, and discussed in this work. These described activities and their possible roles discussed here are very useful to understand the mechanisms involved in the toxic effects in the first, and probably one of the most important stages, of *M. anisopliae* host infection process.

Acknowledgments

This work was supported by grants and fellowships from: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Programa de Aperfeiçoamento Pessoal de Nível Superior (CAPES).

References

Bagga, S., Hu, G., Screen, S.E., St Leger, R.J., 2004. Reconstructing the diversity of subtilisins in the pathogenic fungus *Metarhizium anisopliae*. Gene 324, 159-169.

Berger, M., Pinto, A.F.M., Guimarães, J.A., 2008. Purification and functional characterization of bothrojaractivase, a prothormbin-activating metalloproteinase isolated from *Bothrops jararaca* snake venom. Toxicon 51 (4), 488-501.

Berto, P., Comménil, P., Belingheri, L., Dehorter, B., 1999. Occurrence of a lipase in spores of *Alternaria brassicicola* with a crucial role in infection of cauliflower leaves. FEMS Microbiol. Lett. 180 (2), 183-189.

Beyer, W.F., Fridovich, I., 1987. Assaying for superoxide dismutase activity: Some large consequences of minor changes in conditions. Anal. Biochem. 161, 559-566.

Bogo, M.R., Rota, C.A., Pinto Jr, H., Ocampos, M., Correa, C.T., Vainstein, M.H., Schrank, A., 1998. A chitinase encoding gene (chit1 gene) from the entomopathogenic *Metarhizium anisopliae*: isolation and characterization of genomic and full-length cDNA. Curr. Microbiol. 37 (4), 221-225.

Cakmak, I., Marschner, H., 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. Plant Physiol. 98 (4), 1222-1227.

Charnley, A.K., 2003. Fungal pathogens of insects: Cuticle degrading enzymes and toxins. Adv Bot Res. 40, 241-321.

Dani, M.P., Edwards, J.P., Richards, E.H., 2005. Hydrolase activity in the venom of the pupal endoparasitic wasp, *Pimpla hypochondriaca*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 141 (3), 373-81.

Davies, K.A., De Lorono, I., Foster, S.J., Li, D., Johnstone, K., Ashby, A.M., 2000. Evidence for a role of cutinase in pathogenicity of *Pyrenopeziza brassicae* on brassicas. Phys. Mol. Plant Pathol. 57, 63-75.

Diaz, P., Prim, N., Javier Pastor, F.I., 1999. Direct fluorescence based lipase activity assay. Biotechniques 27 (4), 696-700.

Donatti, A.C., Furlaneto-Maia, L., Fungaro, M.H., Furlaneto, M.C., 2008. Production and regulation of cuticle-degrading proteases from *Beauveria bassiana* in the presence of *Rhammatocercus schistocercoides* cuticle. Curr. Microbiol. 56 (3), 256-260.

Fan, Y., Fang, W., Guo, S., Pei, X., Zhang, Y., Xiao, Y., Li, D., Jin, K., Bidochka, M.J., Pei, Y., 2007. Increased insect virulence in *Beauveria bassiana* strains overexpressing an e engineered chitinase. Appl. Environ. Microbiol. 73 (1), 295-302.

Frazzon, A.P.G., Da Silva Vaz Junior, I., Masuda, A., Schrank, A., Vainstein, M.H., 2000. *In vitro* assessment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus*. Vet. Parasitol. 94 (1-2),117-125.

Freimoser, F.M., Hu, G., St Leger, R.J., 2005. Variation in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation *in vitro.* Microbiol. 151 (2), 361-371.

Garre, V., Tenberge, K.V., Eising, R., 1998. Secretion of a fungal extracellular catalase by *Claviceps purpurea* during infection of rye: putative role in pathogenicity and suppression of host defense. Phytopathol. 88 (8), 744-753.

Ghannoum, M.A., 2000. Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev. 13 (1), 122-143.

Gillespie, J.P., Bateman, R., Charnley, A.K., 1998. Role of cuticle-degrading proteases in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria.* J. Invert. Pathol. 71 (2), 128-137.

Göttlich, E., de Hong, G.S., Yoshida, S., Takeo, K., Nishimura, K., Miyaji, M., 1995. Cell-surface hydrophobicity and lipolysis as essential factors in human tinea nigra. Mycoses 38 (12-13), 489-494.

Ibrahim, A.S., Mirbod, F., Filler, S.G., Banno, Y., Cole, G.T., Kitajima, Y., Edwards, J.E., Nozawa, Y., Ghannoum, M.A., 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. Infect. Immun. 63 (5), 1993-1998.

Joshi, L., St Leger, R.J., 1999. Cloning, expression, and substrate specificity of MeCPA, a zinc carboxypeptidase that is secreted into infected tissues by the fungal entomopathogen *Metarhizium anisopliae*. J. Biol. Chem. 274 (14), 9803-11.

Krieger de Moraes, C., Schrank, A., Vainstein, M.H., 2003. Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. Curr. Microbiol. 46 (3), 205-10.

Kurioka, S., Matsuda, M., 1976. Phospholipase C assay using p-nitrophosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. Anal. Biochem. 75, 281-289.

Liu, Y., Wag, Z., Yin, Y., Cao, Y., Zhao, H., Xia, Y., 2004. Expression, purification, and characterization of recombinant *Metarhizium anisopliae* acid trehalase in *Pichia pastoris*. Protein Expr. Purif. 54 (1), 66-72.

Pedrini, N., Crespo, R., Juarez, M.P., 2007. Biochemistry of insect epicuticle degradation by entomopathogenic fungi. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146 (1-2), 124-37.

Pinto, A.F.M., Silva, K.R.L.M., Guimarães, J.A., 2006. Proteases from *Lonomia obliqua* venomous secretions: Comparison of procoagulant, fibrin(ogen)olytic and amidolytic activities. Toxicon 47 (1), 113-121.

Qazi, S.S., Khachatourians, G.G., 2007. Hydrated conidia of *Metarhizium anisopliae* release a family of metalloproteases. J. Invert. Pathol. 95 (1), 48-59.

Quesada-Moraga, E., Santos-Quirós, R.,Valverde-García, P., Santiago-Álarez, C., 2004. Virulence, horizontal transmission, and sublethal reproductive effects *of Metarhizium anisopliae* (Anamorphic fungi) on the German cockroach (Blattodea: Blattellidae). J. Invertebr. Pathol. 87 (1), 51-58.

Rangel, D.E.N., Braga, G.U., Flint, S.D., Anderson, A.J., Roberts, D.W., 2004. Variations in UV-B tolerance and germination speed of *Metarhizium anisopliae* conidia produced on insects and artificial substrates. J. Invertebr. Pathol. 87 (2-3), 77-83.

Rangel, D.E.N., Alston, D.G., Roberts, D.W., 2008. Effects of physical and nutritional stress conditions during mycelial growth on conidial germination speed, adhesion to host cuticle, and virulence of *Metarhizium anisopliae*, an entomopathogenic fungus. Mycol. Res. 112 (11), 1355-1361.

Roberts, D.W., St Leger, R.J., 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. Adv. Appl. Microbiol. 54, 1-70.

Salyers, A., Witt, D., 1994. Virulence factors that promote colonization. In: Salyers, A., Witt, D. (Ed.) Bacterial pathogenesis: a molecular approach. ASM press, Washington, D.C, pp.30-46.

Schofield, D.A., Westwater, C., Warner, T., Balish, E., 2005. A diferential *Candida albicans* lipase gene expression during alimentary tract colonization and infection. FEMS Microbiol. Lett. 244 (2), 359-365.

Scholte, E-J., Ng'habi, K., Kihonda, J., Takken, W., Paaijman, K., Abdulla, S., Killeen, G.F., Knols, B.G.J., 2005. An entomopathogenic fungus for control of adult african malaria mosquitoes. Science 308 (5728), 1641-1642.

Schrank, A., Bassanesi, M.C., Pinto Jr, H., Costa, S.V., Bogo, M.R., Silva, M.S.N., 1993. Superoxide dismutases in the entomopathogenic fungus *Metarhizium anisopliae*. Ciência e Cultura 45, 200-205.

Screen, S.E., St Leger, R. J., 2000. Cloning, expression, and substrate specificity of a fungal chymotrypsin. Evidence for lateral gene transfer from an actinomycete bacterium. J. Biol. Chem. 275 (9), 6689-6694.

Selvaggini, S., Munro, C.A., Paschoud, S., Sanglard, D., Gow, N.A., 2004. Independent regulation of chitin synthase and chitinase activity in *Candida albicans* and *Saccharomyces cerevisiae*. Microbiol. 150 (4), 921-928.

Shah, P.A., Pell, J.K., 2003. Entomopathogenic fungi as biological control agents. Appl. Microbiol. Biotechnol. 61 (5-6), 413- 423.

Shah, F.A., Allen, N., Wright, C.J., Butt, T.M., 2007. Repeated in vitro subculturing alters spore surface properties and virulence of *Metarhizium anisopliae*. FEMS Microbiol. Lett. 276 (1), 60-66.

Silva, M.V., Santi, L., Staats, C.C., Costa, A.M., Colodel, E.M., Driemeier, D., Vainstein, M.H., Schrank, A., 2005. Cuticle-induced endo/exoacting chitinase CHIT30 from *Metarhizium anisopliae* is encoded by an ortholog of the *chi3* gene. Res. Microbiol. 156 (3), 382-392.

Silva, W.O.B., Mitidieri, S., Schrank, A., Vainstein, M.H., 2005. Production and extraction of an extracellular lipase from the entomopathogenic fungus *Metarhizium anisopliae*. Proc. Biochem. 40 (1), 321-326.

Small, C.L.N., Bidochka, M.J., 2005. Up-regulation of Pr1, a subtilisin-like protease, during conidiation in the insect pathogen *Metarhizium anisopliae*. Mycol. Res. 109 (3), 307-313.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., et al., 1985. Measurement of the principle of protein-dye binding. Anal. Biochem. 150, 76-85.

St Leger, R.J., Staples, R.C., Roberts, D.W., 1993. Entomopathogenic isolates of *Metarhizium anisopliae, Beauveria bassiana,* and *Aspergillus flavus* produce multiple extracellular chitinase isozymes. J. Invert. Pathol. 61, 81-84.

St Leger, R.J., Joshi, L., Bidochka, R.J., Rizzo, N.W., Roberts, D.W., 1996a. Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Appl. Env. Microbiol. 62 (4), 1257-1264.

St Leger, R.J., Joshi, L., Bidochka, M.J., Rizzo, N.W., Roberts, D.W., 1996b. Characterization and ultrastructural localization of chitinases from *Metarhizium*

anisopliae, M. flavoviride, and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Appl. Environ. Microbiol. 62 (3), 907-912.

St Leger, R.J., Joshi, L., Roberts, D.W., 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Appl. Env. Microbiol. 64 (2), 709-713.

Stehr, F., Kretschmar, M., Kroger, C., Hube, B., Schafer, W., 2003. Microbial lipases as virulence factors. J. Mol. Cat. B: Enzym. 22 (5-6), 347-355.

Tolfo Bittencourt, S.E., Castro, L.A., Farias, S.E., Bao, S.N., Schrank, A., Vainstein, M.H., 2004. Purification and ultrastructural localization of a copper-zinc superoxide dismutase (CuZnSOD) from the entomopathogenic and acaricide fungus *Metarhizium anisopliae*. Res. Microbiol. 155 (8), 681-687.

Vieira, I.C., Lupetti, K.O., Fatibello-Filho, O., 2003. Determination of paracetamol in pharmaceutical products using a carbon paste biosensor modified with crude extract of zucchini (*Cucurbita pepo*). Quimica Nova 26, 39-43.

Voigt, C.A., Schafer, W., Salomon, S., 2005. A secreted lipase of *Fusarium graminearum* is a virulence factor required for infection of cereals. Plant J. 42 (3), 364-375.

Xia, Y., Clarkson, J.M., Charnley, A.K., 2002. Trehalose-hydrolysis enzymes of *Metarhizium anisopliae* and their role in pathogenesis of the tobacco hornworm, *Manduca sexta*. J. Invert. Pathol. 80 (3), 139-147.

Zhang, Y., Liu, X., Wang, M., 2008. Cloning, expression, and characterization of two novel cuticle-degrading serine proteases from the entomopathogenic fungus *Cordyceps sinensis*. Res. Microbiol.159 (6), 462-469.

Zimmermann, P., Heinlein, C., Orendi, G., Zentgraf, U., 2006. Senescence-specific regulation of catalases in *Arabidopsis thaliana* (L.) Heynh. Plant Cell Environ. 29 1049-1060.

Fig. 1 SDS-PAGE and zymograms of *Metarhizium anisopliae* spore surface proteins. (A) SDS-PAGE 12%; (B) zymograms of protease (Pro) in 12% gel with gelatin as substrate, superoxide dismutase (SOD) in 8.5% gel with NBT as substrate, lipase (Lip) in 10% native gel with MUF-butyrate as substrate, catalase (Cat) in 7.5% native gel with hydrogen peroxide as substrate, and chitinase (Chi) in 12% gel with glycol chitin as substrate. The gels were loaded with 50 μg of protein.

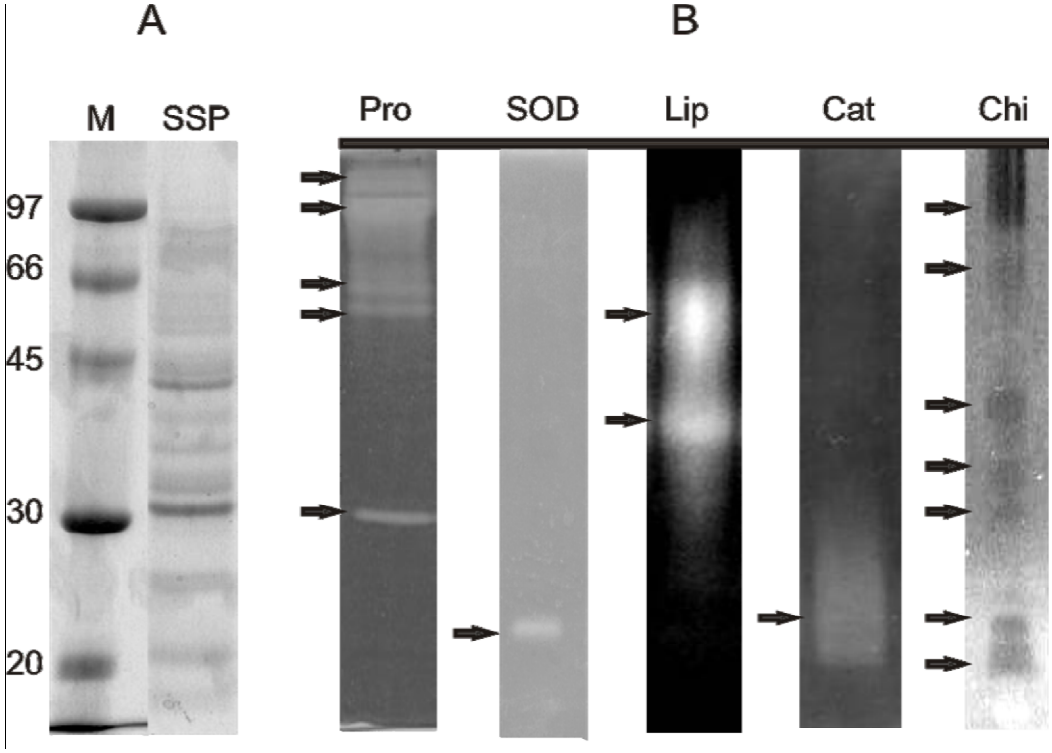


Table 1 Enzymatic activities present in spore surface proteins.

Enzyme	Activity (U)
Protease	
BAPNA (benzoyl-DL-arginine-pNA)	0.076 ± 0.0079
Suc-ala-ala-pro-phe-pNA	16.23 ± 3.98
(S-2302) HD-Pro-phe-arg-pNA	0.92 ± 0.034
(S-2366) Pir-glu-pro-arg-pNA	0.84 ± 0.06
(S-2251) HD-val-leu-lys-pNA	0.289 ± 0.008
(S-2238) HD-phe-pip-arg-pNA	1.2 ± 0.12
(S-2222) Bz-ile-glu-gly-arg-pNA	1.62 ± 0.06
(S-2160) Bz-phe-val-arg-pNA	4.97 ± 0.47
(S-2337) Pip-gly-arg-pNA	2.32 ± 0.091
Chitinase	
$4MU_4$	42.6 ± 2.97
$4MU_3$	37.5 ± 1.24
$4MU_2$	45.87 ± 2.18
Lipase	
pNP-palmitate	0.98 ± 0.055
pNP-myristate	0.489 ± 0.021
Lipase / sterase	
pNP-butyrate	29.24 ± 0.8
pNP-acetate	10.62 ± 0.169
Phospholipase C	0.429 ± 0.021
Trehalase	182.9 ± 7.37
Peroxidase	420 ± 0.32
Superoxide dismutase	302.6 ± 18.95
Catalase	2418 ± 22.21

pNA- p-nitroaniline; 4MU- 4-methylumbelliferyl; pNP- p-nitrophelyl

± Standard deviation (based on three replicates).

Table 2 Effect of metals and protease inhibitors on activity of *M. anisopliae* conidial surface proteins.

Reagent (concentration)	Residual activity (%)	
	Pr1 substrate*	Pr2 substrate**
Control	100	100
$CaCl_2$ (5 mM)	101 ± 1.77	70 ± 0.50
$ZnCl_2$ (5 mM)	13 ± 0.35	32 ± 0.34
$CoCl_2$ (5 mM)	80 ± 3.78	76 ± 1.35
$MnCl_2$ (5 mM)	99 ± 1.05	66 ± 1.78
$MgCl_2$ (5 mM)	105 ± 2.02	75 ± 0.58
$CuCl_2$ (5 mM)	5 ± 0.22	8 ± 0.15
$BaCl_2$ (5 mM)	118 ± 3.20	79 ± 0.15
$HgCl_2$ (1 mM)	70 ± 0.51	12 ± 0.02
PMSF (10 mM)	0	0
Pepstatin (10 μM)	161 ± 0.76	95 ± 0.35
EDTA (5 mM)	196 ± 2.41	93 ± 1.20
EGTA (5 mM)	192 ± 2.97	109 ± 2.52

* Suc-ala-ala-pro-phe-pNA

** Bz-phe-val-arg-pNA

± Standard deviation (based on four replicates)

4.3. Capítulo III – "Differential immunoproteomics allows the identification of *Metarhizium anisopliae* proteins related with *Rhipicephalus microplus* infection"

Manuscrito submetido a Research in Microbiology

M. anisopliae é fungo entomopatogênico, e sua patogenicidade depende da secreção de um complexo de enzimas hidrolíticas capazes de degradar a cutícula dos hospedeiros. Neste trabalho, foram identificadas proteínas secretadas por *M. anisopliae* induzidas por carapaça do carrapato bovino, *R. microplus*, por imunoproteômica. Além disso, anticorpos produzidos contra proteínas da superfície do esporo foram utilizados para identificar proteínas envolvidas na fase inicial da patogenicidade. Diferenças significantes foram detectadas em mapas de *western blot* bidimensional (2DE), usando os diferentes antisoros produzidos. *Spots* detectados com antisoro produzido de sobrenadante de cultura do fungo em meio contendo glicose foram excluídos, a fim de identificar proteínas envolvidas no processo de infecção. LC-MS/MS de proteínas diferencialmente secretadas permitiu a identificação de duas proteases (carboxipeptidase e Pr1A), uma quitinase, uma proteína transportadora de ácido carboxílico e uma proteína rica em prolina. Interessantemente, algumas proteínas identificadas neste trabalho utilizando antisoros não foram visualizadas com corantes usuais de coloração de proteínas, indicando a sensibilidade e a eficiência da estratégia aplicada.

Your manuscript has been assigned the following ID: [res090079].

The information we have received has been forwarded by e-mail to the editor at Research in Microbiology.

Access the author account which will let you

- upload your figures
- view or update all your submitted information
- know the current refereeing status of your submission

Please keep this message in a safe place, you will need this information for future online registrations or changes.

Yours sincerely,

Institut Pasteur

Differential immunoproteomics allows the identification of *Metarhizium anisopliae* proteins related with *Rhipicephalus microplus* infection

Lucélia Santi [a], Walter Orlando Beys da Silva [a], Antônio Frederico Michel Pinto [b], Augusto Schrank [a,c], Marilene Henning Vainstein [a,c]*

[a] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, P. O. Box 15005, CEP 91501-970, Porto Alegre, RS, Brazil,

[b] Department of Microbiology, University of Virginia, P.O. Box 800734, Charlottesville, VA 22908-0734, USA

[c] Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, P. O. Box 15005, CEP 91501-970, Porto Alegre, RS, Brazil,

*Corresponding author. Centro de Biotecnologia and Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, P. O. Box 15005, CEP 91501-970, Porto Alegre, RS, Brazil. Phone +55 (051) 3308-6060 and Fax +55 (051) 3308-7309. mhv@cbiot.ufrgs.br

Abstract

This paper used the differential immunoproteomic technique to identify the proteins secreted by *M. anisopliae* induced by *Rhipicephalus microplus* cuticle. Also, IgG anti spore surface proteins were used to identify proteins possibly involved in the early stages of infection. LC-MS/MS of the differentially secreted proteins allowed the identification of proteases (carboxypeptidase and Pr1A), chitinase, carboxylic acid transport, and proline-rich protein. The differential immunoproteomics strategy used facilitated the detection and the identification of new proteins related with *M. anisopliae* host-pathogen interaction and could be applied in future works to identify novel proteins related to other microbial infection systems.

Keywords: Immunoproteomics; *Metarhizium anisopliae*; *Rhipicephalus microplus*; Host-pathogen interaction.

1. Introduction

The filamentous fungus *Metarhizium anisopliae* is one of the most important, applied and studied agents used in the biological control of many arthropod plagues [8] including the cattle tick *Rhipicephalus microplus* that causes considerable losses in leather, meat, and milk production [3]. To penetrate the host cuticles, *M. anisopliae* utilizes a synergistic strategy of secretion of cuticle-degrading enzymes [8] associated with mechanical pressure (apressorium).

In comparison to enzymatic activities described for *M. anisopliae*, little is known about the infective system secreted by this fungus, despite the fact that it can also play a significant role in the biocontrol activity for specific host-pathogen relationships. The chemical composition of the host cuticle is believed to induce the secretions of different proteins for diverse hosts.

Proteomics have been receiving increased attention, since this technique is utilized to identify new proteins involved in several processes, like mycoparasitism [15]. Also, immunoproteomics, a technique involving 2D electrophoresis followed by immunoblotting, helps to identify immunogenic proteins, as previously reported [17].

In the present work, we have carried out a differential immunoproteomic approach to reveal the differences in proteins secreted by *M. anisopliae* in culture media induced by tick cuticle for the infection condition. This methodology allowed the detection and identification of proteins possibly involved in tick infection process. In addition, spore surface proteins were used to identify proteins possibly present at the initial stage of pathogenicity or infection.

2. Materials and methods

2.1. Culture conditions, extraction of spore surface proteins (SSP) and cuticle preparation

M. anisopliae var. *anisopliae* isolate E6 from spittlebug (Deois flavopicta) from the state of Espírito Santo, Brazil was kept and conidia for liquid culture growth were produced as previously described [10].

To mimic infection conditions, spores (10^6 mL^{-1}) were inoculated in minimal medium (MM; 0.1% KH_2PO_4 and 0.05% $MgSO_4$) containing 0.8% *R. microplus* (RM) cuticle or 0.8% glucose (G). The flasks were incubated at 28 ^oC with shaking (150 rpm). After 96-h incubation, mycelia were harvested by filtration through a Whatman No. 1 filter paper and the culture filtrate used for experiments.

The protein content was determined using the method described by Bradford [1].

M. anisopliae spore production and the extraction procedure of spore surface proteins were performed as previously described [11]. The resulting supernatant of the extraction procedure was called spore surface proteins (SSP).

R. microplus engorged females were crushed by pressing to remove internal material. The cuticles were rinsed extensively 5 times with sterile distilled water and sterilized by autoclaving before use.

2.3. Antiserum production and IgG purification

Antiserum against G supernatants and against SSP was produced in 2-month-old New Zealand rabbits. Supernatants emulsified with Freund's incomplete adjuvant were injected subcutaneously, at 7-day intervals (total of 4 injections). Five weeks after the first immunization, blood was collected and serum samples were evaluated by ELISA and western blot. Sera collected prior to immunization were used as control. The produced sera were purified in HiTrap Protein G HP (GE) according the manufacturer's instructions.

2.4. One and two dimensional gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [6]. Then 1DE was performed on vertical 12% polyacrylamide (0.75 mm thickness) gel. For 2DE, protein samples were solubilized in isoelectric focusing buffer (IEF buffer) containing 7 M urea, 2 M thio-urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT) and 0.2% (v/v) ampholytes pH 3–10 (Bio-Rad). The 7-cm immobilized pH gradient (IPG) strips (pH 3–10 linear, Bio-Rad) were passively rehydrated for 16 h with 150 μg of protein. Isoelectric focusing was performed in a Protean IEF cell system (Bio-Rad) with up to 10.000 Vh at a maximum voltage of 4.000 V. Strips were equilibrated for 15 min in equilibration buffer I containing 1% DTT and for 15 min in equilibration buffer II containing 4% iodoacetamide. In the second dimension, IPG strips were run vertically onto SDS-PAGE 12% gels using PROTEAN II Cell (Bio-Rad). The 1DE and 2DE gels were subsequently stained with Coomassie

Brilliant Blue R250 (Bio-Rad). Stained 2DE gels were scanned with an Image Scanner (Amersham Biosciences) and analyzed with the PDQuest Basic-8.0 software (Bio-Rad). The gels were evaluated in biological triplicate.

2.5. Immunoblotting

For western blot analysis, proteins of *M. anisopliae* cultured in MM plus RM cuticle were determined by 2DE and electroblotted onto nitrocellulose membranes (GE). Blotted membranes were blocked with 5% (w/v) nonfat dried milk in PBS (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl), containing 0.05 % Tween 20 and stored overnight at a 4 ^oC. They were washed 3 times for 10 min with PBS containing Tween; then incubated with the rabbit anti-G or anti-SSP IgG diluted both 1:1000, in blocking solution. Membranes were washed three times in PBS for 10 min, incubated with a secondary antibody (anti-rabbit IgG alkaline phosphatase conjugate, Sigma) (1:2000), washed and developed with NBT/BCIP (Sigma) according to the manufacturer's instructions. Western blots were evaluated in technical duplicate, and statistically significant changes were determined using two sequential data analysis criteria. First, a spot had to be present in all immunoblots for a sample to be included in the analysis. Next, two sample *t* tests were used to analyze differences in immunodetection between samples. *P*-values under 0.05 were considered statistically significant.

2.6. Protein sequencing

To analyze protein fragments, 2DE separated proteins were stained and the spot cut out of the gel. The proteins were digested in gel with modified trypsin (Promega) as previously described [12]. Mass spectrometric analysis of peptides was performed with an LTQ XL instrument (Thermo Electron Corporation, Waltham, MA). Data were collected in a top 10 mode, which means that one MS scan was taken and followed by 10 MS/MS fragmentation spectra of the top intensity ions collected. After MS/MS fragmentation, the m/z of a particular parent ion was placed on an exclusion list. The repeat count for the exclusion list was 1, repeat duration was 30 seconds and exclusion duration was 120 seconds. The electrospray voltage was set to 2.5 kV, capillary temperature was 230 °C and flow rate was 500 nl min^{-1}.

The mass spectra were extracted and analyzed utilizing Bioworks Sequest 3.11. A database containing *M. anisopliae* proteins and nucleotide sequences was used. Spectra were searched using 1.5-Da parent tolerances and 1-Da fragment tolerance. All hits were required to be fully tryptic.

3. Results

Proteins secreted by *M. anisopliae* in different culture media and spore surface proteins were separated on 1DE gels (Fig. 1). The conditions of induced, non-induced (glucose as carbon source), and *R. microplus* cuticle alone (negative control) showed clear differences in secreted protein profiles.

The 2DE gels of proteins secreted in culture containing tick cuticle had spots primarily in the region between 20 – 66 kDa and pI 5 – 9 stained with Coomassie (Fig. 2a). Approximately 35 spots were observed in gels with different pIs and molecular masses. Figure 2d shows the negative control, (*M. anisopliae* cultured in MM containing glucose). Spots of proteins present and immunodetected with IgG anti-SSP (Fig. 2b), but not present in media containing glucose (Fig. 2c), were excised from gel. Following in gel trypsin digestion, peptides were identified on LC-MS/MS. In 2DE, we identified proteins involved in cuticle degradation, including proteases (spots 2, 3, 4 and 5) and one chitinase (spot 6). Also, a carboxylic acid transport protein (spots 1 and 8) and a proline-rich protein (spot 7) (Table 1) was identified. Interestingly, spots 1, 4, and 8 were not visible in Coomassie stain, but only when immunodetection was used. All spots identified in Table 1 are recognized by IgG anti-SSP and not by IgG anti-G, having been differentially secreted in infection induction condition.

4. Discussion

The present study applied a methodology that we named differential immunoproteomics to detect and identify proteins potentially associated with the infection process on *R. microplus* tick by *M. anisopliae*.

When comparing the 1DE gels, there were clear differences in secreted protein profile from *M. anisopliae* growth in media that mimic infection conditions (*R. microplus* cuticle) and in SSP, compared to non-induction media (glucose) and *R. microplus* cuticle alone. This difference in protein secreted profile supports the fact that certain proteins are

expressed in response to the induction of the host's cuticle, which has also observed in other fungus [15].

Proteases are fundamental in the host-infection process and were described for *M. anisopliae* during arthropod pathogenesis [13]. In addition, no other fungal species has been reported to have such a diverse array of proteases as *M. anisopliae* [4]. Here, we detected two different proteases (carboxypeptidase or elastase-like serine protease and Pr1A). Recently, in EST analysis of two *M. anisopliae* strains gene expressions, an upregulation of corresponding carboxyprotease and *pr1a* genes in media containing cuticles have been detected [4]. Blast results showed similarity among carboxyprotease of other microbial pathogens as *Trichophyton rubrum*, a human dermatophyte [18], and serine protease of *Pochonia chlamydosporia* [7], which reinforces the involvement of these enzymes in pathogenicity. These findings make it clear that proteases are really important for insect colonization by pathogenic fungus and those different proteases are involved in infection of different hosts.

Chitinases are also important for host-pathogen interaction by degrading chitin content in arthropod cuticles, and could be identified in extracellular secretions of *M. anisopliae* [10]. Spot 6 was identified as chitinase from *M. anisopliae* var *acridum,* described previously [9], and that overexpression does not enhance virulence against *Manduca sexta*. Another chitinase with similar mass (43.5 kDa) was detected in media containing cockroach cuticle, and in immunodetection. Which suggests that this chitinase is probably important for *R. microplus* infection, being host-selective, also reported for Pr1A [14]. There could also exist a synergistic effect of pathogenicity factors and the hydrolases diversity maybe important.

Carboxylic acid transporter protein was identified in our study. Interestingly, this spot was not stained with Coomassie, but was detected with antiserum. The gene that codifies this transport protein was upregulated when *M. anisopliae* was grown in media containing three different cuticles [4]. Also, the study of the function of one protein with homology to carboxylic acid transport protein from *Pectobacterium atrosepticum* revealed that the protein is cryptic for full virulence in potato [16]. The proline-rich protein could perform several functions, including protein-protein interactions and stress response [5]. These proteins could play a role as co-regulator of pathogenicity, as previously reported for other proteins that do not directly act in infection [4]. Therefore, these results provide additional rich resources for future studies with new approach in *M. anisopliae* infection.

The differential immunoproteomic technique identified immunogenic proteins secreted by *M. anisopliae* present in *in vitro* infection conditions and in conidial surface with putative roles in infection. This is the first report which uses this differential technique to identify proteins in host-pathogen interaction. This technique enabled the identification of low abundant proteins, only detectable by antibody recognition, providing a new important source of novel factors involved in pathogenicity.

Acknowledgements

We thank Dr. Jay W. Fox and Dr. Nicholas E. Sherman from Microbiology Department, University of Virginia for their assistance with the mass spectrometric analyses. This work was supported by grants and fellowships from Conselho Nacional de

Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

[1] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye biding. Anal. Biochem. 72, 248–254.

[2] Dutra, V., Nakazato, L., Broetto, L., Schrank, I.S., Vainstein, M.H., Schrank, A. (2004) Application of representational difference analysis to identify sequence tags expressed by *Metarhizium anisopliae* during the infection process of the tick *Boophilus microplus* cuticle. Res. Microbiol. 155, 245–251.

[3] Frazzon, A.P.G., Da Silva Vaz Junior, I., Masuda, A., Schrank, A., Vainstein, M.H. (2000) *In vitro* assessment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus*. Vet. Parasitol. 94, 117–125.

[4] Freimoser, F.M., Hu, G., St Leger, R.J. (2005) Variation in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation *in vitro*. Microbiol. 151, 361–371.

[5] He, C.Y., Zhang, J.S., Chen, S.Y. (2002) A soybean gene encoding a proline-rich protein is regulated by salicylic acid, an endogenous circadian rhythm and by various stresses. Theor. Appl. Genet. 104, 1125–1131.

[6] Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680–685.

[7] Morton, C.O., Hirsch, P.R., Peberdy, J.P., Kerry, B.R. (2003). Cloning of and genetic variation in protease VCP1 from the nematophagous fungus *Pochonia chlamydosporia*. Mycol. Res. 107, 38–46.

[8] Roberts, D.W., St Leger, R.J. (2004) *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. Adv. Appl. Microbiol. 54, 1–70.

[9] Screen, S.E., Hu, G., St Leger, R.J. (2001) Transformants of *Metarhizium anisopliae* sf. *anisopliae* overexpressing chitinase from *Metarhizium anisopliae* sf. *acridum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta.* J. Invertebr. Pathol. 78, 260–266.

[10] Silva, M.V., Santi, L., Staats, C.C., Costa, A.M., Colodel, E.M., Driemeier, D., Vainstein, M.H., Schrank, A. (2005) Cuticle induced endo/exoacting chitinase CHIT30 from *Metarhizium anisopliae* is encoded by an ortholog of the *chi3* gene. Res. Microbiol. 156, 382–392.

[11] Silva, W.O.B., Santi L., Berger, M., Pinto, A.F.M., Guimarães, J.A., Schrank, A., Vainstein, M.H. (2009) Characterization of a spore surface lipase from the biocontrol agent *Metarhizium anisopliae*. Proc. Biochem. doi:10.1016/j.procbio.2009.03.019.

[12] Shevchenko, A., Wilm, M., Vorm, O., Mann, M. (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858.

[13] St Leger, R.J., Joshi, L., Bidochka, R.J., Rizzo, N.W., Roberts, D.W. (1996a) Biochemical characterization and ultrastructural localization of two extracellular

trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Appl. Environ. Microbiol. 62, 1257–1264.

[14] St Leger, R.J., Joshi, L., Bidochka, M.J., Roberts, D.W. (1996c) Construction of an improved mycoinsecticide overexpressing a toxic protease. Proc. Natl. Acad. Sci. USA. 93, 6349–6354.

[15] Suarez, M.B., Sanz, L., Chamorro, M.L., Rey, M., González, F.J., Llobell, A., Monte, E. (2005) Proteomic analysis of secreted proteins from *Trichoerma harzianum*: identification of a fungal cell wall-induced aspartic protease. Fungal Genet. Biol 42, 924–934.

[16] Urbany, C., Neuhaus, H.E. (2008) Citrate uptake into *Pectobacterium atrosepticum* is critical for bacterial virulence. Mol. Plant Microbe Interact. 21, 547–554.

[17] Wu, Z., Zhang, W., Lu, C. (2008) Immonoproteomic assay of surface proteins of *Streptococcus suis* serotype 9. FEMS Immunol. Med. Microbiol. 53, 52–59.

[18] Zaugg, C., Jousson, O., Léchenne, B., Staib, P., Monod, M. (2008) *Trichophyton rubrum* secreted and membrane-associated carboxypeptidases. Int. J. Med. Microbiol. 298, 669–682.

Table 1. Results of spot identification of *M. anisopliae in vitro* infection conditions using immunoproteomic technique.

Spot no.	Identification	Accession no.	Theoretical pI/mass (kDa)	Observed pI/mass (kDa)	Sequence (unic peptides)
1	Carboxylic acid transport protein (*M. anisopliae*)	gi|21928180	9.29/56105	3.3/16	KVAGMTSEESSTKVMESEDEEKG
2	Carboxypeptidase (*M. anisopliae*)	gi|197253670	6.59/45953	Nd/40	RGASAGNSPEVKS RNQGSSCLGTDPNRN KSYIAFLDKI RTPNNAALQALAKG KWDGPGSSTNPCTETYRG
	Elastase-like serine protease (*M. anisopliae*)	gi|16506140	8.27/37340		RDAQNTSPASEPSACTVGATDSSDRRS KNALTGVPSGTVNYLAYNGNGA RRSSFSNFGRV
3	Carboxypeptidase (*M. anisopliae*)	gi|197253670	6.59/45953	7.5/40	KADNVFTIELRD RNQGSSCLGTDPNRN RSVHGTTFAYGPVCNVIYQVAGGSIDWVQDVLKA KSYIAFLDKI RTPNNAALQALAKG

					KWDGPGSSTNPCTETYRG
	Elastase-like serine protease (*M. anisopliae*)	gi|16506140	8.27/37340		RDAQNTSPASEPSACTVGATDSSDRRS KNALTGVPSGTVNYLAYNGNGA- RSFISGQETDGHGHGTHCAGTIGSKS
4	Subtilisin-like serine protease PR1A (*M. anisopliae*)	gi|16215662	7.14/40303	4.6/18	RTDDSAGEGTCVYIIDTGIEASHPEFEGRA
5	Subtilisin-like serine protease PR1A (*M. anisopliae*)	gi|16215662	7.14/40303	6.4/50	RYDDSAGEGTCVYIIDTGIEASHPEFEGRA
6	Chitinase (*M. anisopliae* var. *acridum*)	gi|71494532	6.22/46129	6.2/43	KIVLGMPIYGKS KLGGPDATENLLNYPDSKY RTQELISYDTPDITKE RTWELISYDTPDITKE
7	Proline rich protein 5MeD (*M. anisopliae*)	gi|2352910	4.99/55702	5.1/66	KATLVNSNVNAGPSGSHINAIGFNIRD RDTGSGSNELYEVDLASGKT KVADSTAPVEFIAKG
8	Carboxylic acid transport protein (*M. anisopliae*)	gi|21928180	9.29/56105	4.3/16	KVAGMTSEESSTKVMESEDEEKG

[a] pI and MW theorethical calcutated according ExPASY program. Nd – not determinate

Fig. 1. SDS-PAGE analysis of secreted proteins of *M. anisopliae* in minimal medium supplemented with *R. microplus* cuticle (Rm) or glucose (G). Spore surface proteins (SSP) were also analyzed and minimal medium containing *R. microplus* cuticle alone (C) was used as a negative control. Gels were loaded with 50 μg of protein.

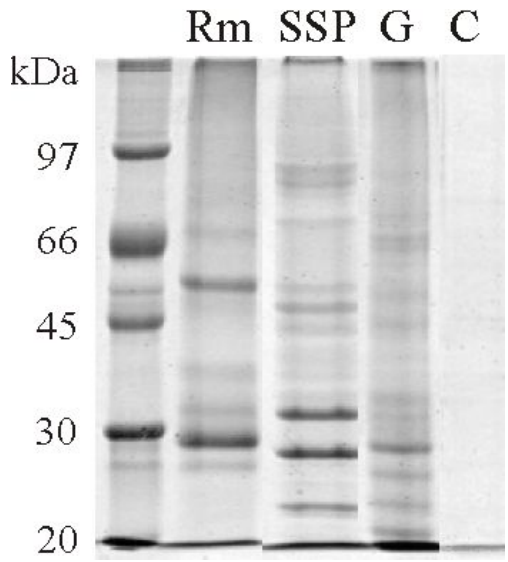
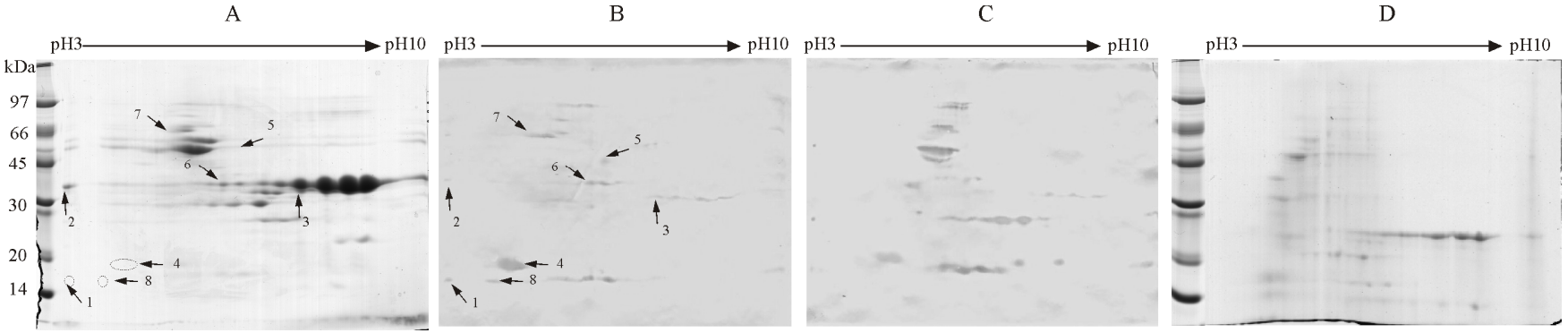


Fig. 2. 2DE map and immunoblot of secreted proteins of *M. anisopliae* cultured in minimal medium supplemented with *R.microplus* cuticle. (A) 2DE gel stained with Coomassie Blue R250. Other gels were run in parallel and used for immunoblotting using (B) anti-SSP (1:1000) or (C) anti-G (1:1000) sera (non induced condition - negative control). (D) 2DE map of secreted proteins of *M. anisopliae* cultured in minimal medium plus glucose. The numbers and arrows indicate analyzed and identified spots by LC-MS/MS.



4.4. Capítulo IV – "*Metarhizium anisopliae* host-pathogen interaction: Differential immunoproteomics reveals proteins involved in the infection process of arthropods"

Manuscrito a ser submetido a FEMS Microbiology Letters

A patogenicidade de *M. anisopliae* sobre hospedeiros artrópodes depende da secreção de enzimas hidrolíticas capazes de degradar a cutícula dos hospedeiros. Para identificar proteínas possivelmente envolvidas no processo de infecção e especificidade por hospedeiros, análise imunoproteômica foi realizada utilizando anticorpos produzidos contra sobrenadante de cultura de *M. anisopliae* na presença de cutículas de *R. microplus* (Arachnida:Acarina) e *D. peruvianus* (Insecta). *Spots* detectados utilizando antisoro produzido contra cutículas e proteínas da superfície do esporo, mas não com antisoro produzido contra sobrenadante do fungo cultivado em meio contendo glicose, foram identificados. O sequenciamento permitiu a identificação de diversas proteases, como elastase, tripsina, quimotripsina, carboxipeptidase e subtilisinas (Pr1A, Pr1I and PR1J), quitinases, DNase 1 e proteína rica em prolina. Quimotripsina e Pr1I foram inferidas como hospedeiro-específicas, sendo reconhecidas apenas em *D. peruvianus*. Este trabalho representa uma contribuição significativa para o entendimento dos mecanismos de adaptação de *M. anisopliae* a diferentes ambientes e hospedeiros.

Metarhizium anisopliae host-pathogen interaction: Differential immunoproteomics reveals proteins involved in the infection process of arthropods

Lucélia Santi[a], Walter Orlando Beys da Silva[a], Antônio Frederico Michel Pinto[b], Jorge Almeida Guimarães[a,c], Augusto Schrank[a,c], Marilene Henning Vainstein[a,c]*

[a] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, P. O. Box 15005, CEP 91501-970, Porto Alegre, RS, Brazil,

[b] Department of Microbiology, University of Virginia, P.O. Box 800734, Charlottesville, VA 22908-0734, USA

[c] Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, P. O. Box 15005, CEP 91501-970, Porto Alegre, RS, Brazil,

* Corresponding author. Centro de Biotecnologia and Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, P. O. Box 15005, CEP 91501-970, Porto Alegre, RS, Brazil. Phone +55 (051) 3308-6060 and Fax +55 (051) 3308-7309. mhv@cbiot.ufrgs.br.

Abstract

Metarhizium anisopliae is an entomopathogenic fungus well characterized for the biocontrol of a wide range of plagues. Its pathogenicity depends on the secretion of hydrolytic enzymes that degrade the host cuticle. To identify proteins involved in the infection process and in host specify, immunoproteomic analysis was performed using antiserum produced against crude extract of *M. anisopliae* cultured in the presence of *Rhipicephalus microplus* and *Dysdercus peruvianus* cuticles. Spots detected using antisera produced against cuticles and spore surface proteins, but not with antiserum anti-glucose, were identified so as to give insights about the infection process. An MS/MS allowed the identification of proteases like elastase, trypsin, chymotrypsin, carboxypeptidase and subtilisin proteases (Pr1A, Pr1I and PR1J), chitinases, DNase 1 and proline-rich protein. Chymotrypsin and Pr1I were inferred as host-specific, being recognized in *D. peruvianus* infection only. This research represents a significant contribution to understand the adaptation mechanisms of *M. anisopliae* to different hosts.

Keywords: Differential immunoproteomic, *Metarhizium anisopliae, Dysdercus peruvianus*, *Rhipicephalus microplus*, DNase 1, Pr1I, Pr1J, Protease.

1. Introduction

In recent years, the use of entomopathogenic fungi has been examined for the biological control of several arthropod pests. The fungus *Metarhizium anisopliae* has been extensively studied and it is the best-characterized entomopathogen used for biological control of several pests, including insects and acari (Kaaya and Munyni, 1995; Frazzon et al., 2000; Quesada-Moraga et al., 2004; Lazzarini et al., 2006; Scholte et al., 2007). *Metarhizium anisopliae* infects arthropods by a combination of the secretion of hydrolytic enzymes, commonly referred to as cuticle-degrading enzymes, and mechanical pressure exerted by the appressorium (Silva et al. 2005; St Leger et al., 1986; Wang and St Leger, 2005). The search for pathogenicity determinants has demonstrated that the process is multifactorial (Dutra et al., 2004; Kershaw et al., 1999; Silva et al., 2005; Silva et al., 2005; St Leger et al., 1996c).

Host specificity is an important aspect for organisms used in biological control, and the study of the proteins involved with them bears significant importance. Studies demonstrate that *M. anisopliae* recognizes specific host signals, like lipids present in the epicuticle and the surface structure, which could affect spore attachment and induce the secretion of different host-specific proteins (Pedrini et al., 2007). By analyzing enzymatic activities described for *M. anisopliae*, it is possible to see that little is known about enzymes and other proteins secreted by this fungus that are specific to host-pathogens.

To identify proteins involved in several physiological processes, including pathogenicity factors and host-specificity, different strategies applying differential proteomics and immunoproteomics are used. Recent works used proteomics to discover

novel compounds with biological control properties (Coumans et al., 2009; Grinyer et al., 2004; Suarez et al., 2005; Wang et al., 2005). Also, immunoproteomic is applied to identify immunogenic proteins in vaccine development (Barbey et al., 2008; Wu et al., 2008), clinical biomarkers (Pedersen et al., 2005) and comparative analysis (Shin et al., 2007). This new methodology using antibodies has made the technique more sensitive, which favors the recognition of proteins present in samples, even at low concentrations.

Using the immunoproteomic approach, that uses two-dimensional electrophoresis (2DE) together with immunoblotting and mass spectrometry, we here investigate the differences in extracellular proteins from *M. anisopliae* that are secreted in the presence of cuticles of two different hosts, the cattle tick *Rhipicephalus microplus* and the cotton stainer bug *Dysdercus peruvianus.* Also, we used proteins secreted in media containing glucose as negative control. This strategy allowed the identification of proteins possibly involved in the infection of specific hosts. Besides, spore surface proteins were used to identify proteins probably present in the initial stage of infection.

2. Material and Methods

2.1. Metarhizium anisopliae and culture conditions

M. anisopliae var. *anisopliae* E6 was kept as previously described (Bogo et al., 1998). Conidia were collected from fungi grown on complete Cove's medium (CM) (Cove, 1966) agar plates in 0.01% Tween 80 solution, washed and maintained in sterile water. Prior to use, the conidia concentration was determined by count using a Neubauer

hemocytometer and suspensions were diluted in sterile water to concentrations used in this work.

To mimic infection conditions, spores (10^6 mL^{-1}) were inoculated in minimal medium (MM; 0.1% KH_2PO_4 and 0.05% $MgSO_4$) containing 0.8% *D. peruvianus* (DP) cuticle, 0.8% *R. microplus* (RM) cuticle or 0.8% glucose (G). The flasks were incubated at 28 ^oC on a rotatory shaking platform (150 rpm). After 96 h incubation, mycelia were harvested by filtration through a Whatman No. 1 filter paper and the culture filtrate used for experiments.

The protein content was determined by the method of Bradford (1976) using known concentrations of bovine serum albumin (BSA) as standard.

2.2. Preparation of cuticles

R. microplus engorged females were crushed for removal internal material. The cuticles were rinsed extensively with sterile distilled water and sterilized by autoclaving before use.

D. peruvianus adults were centrifuged for removal internal content and cleaning with pinças. The cuticles were rinsed and sterilized by autoclaving before use.

2.3. Extraction of spore surface proteins (SSP)

M. anisopliae cultivation for conidia production was performed using 100 g of rice added to 30 mL of a 0.5% peptone solution in polypropylene bags. In each sterilized

bag, 10^6 spores were inoculated and the fungus was grown at 28 °C for 14 days. Sporulated fungus were mechanically desorbed from rice grains by gently shaking using a plastic spatula, and then sieved to collect conidia. The spores obtained were suspended in an extraction buffer (Tris HCl 50 mM pH 8.0 containing 0.25% Triton X-100, 1:2.5 w/v) (Silva et al., 2005). The suspension was vigorously shaken for 5 min and the resulting supernatant was filtered in a 0.2-µm filter (Millipore, USA). The supernatant thus obtained was named spore surface proteins (SSP).

2.4. Antiserum production

Antiserum against DP, RM or G supernatants and against SSP was raised in a two month-old New Zealand white rabbit. Supernatants emulsified with Freund's incomplete adjuvant were injected subcutaneously, at 7 day intervals (total of 4 injections). Five weeks after first immunization, blood was collected and serum samples were evaluated by ELISA. Serum collected prior to immunization was used as control. Serums produced were purified in HiTrap Protein G HP (GE) according manufacture instruction.

2.5. Two dimensional gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method developed by Laemmli (1970). For 2DE, protein samples were solubilized in isoelectric focusing buffer (IEF buffer) containing 7 M urea, 2 M thio-urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT) and 0.2% (v/v) ampholytes

pH 3–10 (Bio-Rad). The 7 cm immobilized pH gradient (IPG) strips (pH 3–10 linear, Bio-Rad) were passively rehydrated for 16 h with 150 µg of protein. Isoelectric focusing was performed in a Protean IEF cell system (Bio-Rad) with up to 10.000 Vh at a maximum voltage of 4.000 V. Strips were equilibrated for 15 min in equilibration buffer I (30%, v/v, glycerol, 6 M urea, 1% DTT, a trace of Bromophenol Blue) and for 15 min in equilibration buffer II (equilibration solution I with DTT replaced by 4% iodoacetamide). In the second dimension, IPG strips were run vertically onto SDS-PAGE 12% gels using PROTEAN II xi Cell (Bio-Rad). Gels were subsequently stained with Coomassie Brilliant Blue R250 (Bio-Rad). The stained gels were scanned with an Image Scanner (Amersham Biosciences) and analyzed with the PDQuest Basic-8.0 software (Bio-Rad). Three replicates were run for each sample.

2.6. Immunoblotting

For immunoblot analysis, proteins of *M. anisopliae* cultured in MM plus DP cuticle are resolved by 2DE and electroblotted onto nitrocellulose membranes (GE). Blotted membranes were blocked with 5% (w/v) nonfat dried milk in PBS (10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) containing 0.05 % Tween 20 (overnight at a 4 ^oC), washed 3 times 10min with PBS containing Tween, and then incubated with the rabbit anti-DP, anti-RM, anti-G or anti-SSP serum diluted 1:1000, 1:2000, 1:1000 and 1:1000, respectively, in blocking solution. Membranes were washed three times in PBS for 10 min, incubated with a secondary antibody (anti-rabbit IgG alkaline phosphatase conjugate, Sigma) (1:2000), washed and developed with NBT/BCIP

(Sigma) according to the manufacturer. For each sample, the western blot was repeated two times.

2.7. Protein sequencing

In order to analyze protein fragments, 2DE separated protein were stained with Coomassie Brilliant Blue and the protein spot of interest cut out of the gel. The proteins were digested in gel with trypsin essentially as described previously (Shevchenko et al., 1996). Mass spectrometric analysis of peptides was performed on a LTQ XL instrument from Thermo Electron Corporation (Waltham, MA). Data was collected in a top 10 mode, meaning one MS scan taken and followed by 10 MS/MS fragmentation spectra of the top intensity ions collected. After MS/MS fragmentation, the m/z of particular parent ion was placed on an exclusion list. The repeat count for the exclusion list was 1, repeat duration was 30 seconds and exclusion duration was 120 seconds. The electrospray voltage was set to 2.5 kV, capillary temperature was 230 °C and flow rate was 500 nL/min.

The mass spectra were extracted and analyzed utilizing Bioworks Sequest 3.11. A database containing *M. anisopliae* proteins and EST sequences was used. Spectra were searched using 1.5 Da parent tolerances and 1 Da fragment tolerance. All hits were required to be fully tryptic.

3. Results

Approximately 110 proteins spots staining with coomassie blue were resolved in the pH 3–10 range of supernatant of *M. anisopliae* cultured in *D. peruvianus* cuticle, with spots prevalence observed in the region between 30–66 kDa and pI 5–9 in 2DE gels (Fig. 1a). The immunoblot profiles revealed 7 antigenic spots identified using anti-SSP, 11 using anti-DP, and 7 using anti-RM antiserum. At least 8 spots were identified using two different antisera.

Spots marked on the gel that are present and immunodetected in the supertantant of media containing *R. microplus* and *D. peruvianus* cuticles and SSP, but not present in media containing glucose, were excised from the gel and, following gel trypsin digestion, peptides were identified on MS/MS (Fig. 1). We could identify proteins involved in cuticle degradation, like proteases, including chymotrypsin (spots 1, 2), subtilisins (spots 5, 6, 7, 8, 9, 22), elastase (spots 11, 12), trypsin (spot 17) and carboxypeptidases (spots 10, 16). Also, we identified chitinases (spots 18, 19), DNase 1 (spot 15) and proline-rich protein (spot 20) (Table 1). Interestingly, chymotrypsin (spots 1, 2) was identified when anti-DP antiserum was used alone. A spot identified as trypsin (spot 17) was detected using anti-SSP antiserum, which can indicate temporal presence in the infection process, as well as host-specificity. Some spots were not visualized in the coomassie blue stain, but only when immunodetection was used.

4. Discussion

The *M. anisopliae* infection process involves the secretion of several enzymes and other associated molecules. Surface structure, topology and the chemical composition of

the host cuticle are both believed to affect the adhesion of fungal spores and, consequently, pathogenicity (Lord and Howard, 2004). *Metarhizium anisopliae* recognizes specific host signals, probably lipids present in the epicuticle, and specificity is the result of these signals (Pedrini et al., 2007). Although many proteins are common in infection of arthropods, we supposed that specific proteins could determine the pathogenicity for specific hosts. Here, we used the differential immunoproteomic methodology to identify proteins involved in pathogenicity and specificity of two different hosts, *R. microplus* and *D. peruvianus*. The immunoproteomic methodology applied in this work gave insights about host-pathogen interactions and specificity.

For pathogenic fungi, it is inferred that the production of enzymes that disrupts the physiological integrity of hosts will have a strong selective advantage for pathogens (Bagga et al., 2004; Bye and Charnley, 2007). For *M. anisopliae*, proteases are fundamental for pathogenicity, since proteins are abundant molecules present in arthropod cuticle (Gillespie et al., 1998; St Leger et al., 1989, 1996a). This fungus secretes a diverse array of proteases, particularly subtilisins, which are fundamental to transpose insect barriers, colonize and digest tissues, and to survive in diverse niches in the environment during saprophytic existence (Freimoser et al., 2003; St Leger et al., 1996c; Wang et al., 2005). The identification of different proteases, including the subtilisins Pr1I and Pr1J, elastase-like serine protease, carboxypeptidase, chymotrypsin and trypsin, reinforces the importance of proteases and the ability of this isolate to cross arthropod cuticle. When the identified subtilisins were analyzed, interesting characteristics were observed: Pr1A was present for both arthropod tested, as well as Pr1J, which also was identified using anti-SSP antiserum, while Pr1I is present only in

DP infection and one isoform identified using anti-SSP. In the microarray analysis, *pr1*J is over-expressed in infection of the cockroach *Blaberus giganteus* and the caterpillar *Lymantria dispar*, but not against the beetle *Popilla japonica*, which indicates that that pathogen can respond in a specific way to different arthropods, producing proteases that are appropriate to degrade a specific arthropod cuticle (Freimoser et al., 2005). Therefore, we can suggest that subtilisins identified here probably play a role in the pathogenesis of the two arthropods tested, but Pr1I seems specific for *D. peruvianus* infection. The subtilisins Pr1I and Pr1J probably were involved in initial stages of the infection process, being found also in the spore surface, differently from Pr1A, which was not detected in spore surface proteins. We also hypothesize that Pr1I could be important to degrade arthropod with soft cuticle, as found in this work for *D. peruvianus* and for the caterpillar *Lymantria dispar*, using microarray (Freimoser et al., 2005).

Trypsins were described in entomopathogenic fungi, like *M. anisopliae*, in response to cockroach (*Periplaneta americana*) (St Leger et al., 1996a) and *Callosobruchus maculatus* (Murad et al., 2008), and in *Zoophthora radicans*, in response to the butterfly *Pieris brassicae* (Xu et al., 2006), reinforcing trypsin participation in pathogenicity. Here, trypsin was identified using anti-SSP antiserum and not recognized when using anti-RM antiserum, suggesting that this protein is not present in *R. microplus* infection but that it is present in the early stages of the pathogenic process of *D. peruvianus*. Freimoser et al. (2005) identified one trypsin gene upregulated only in the presence of cockroach cuticle and not present in other two host cuticles analyzed, supporting the host-specificity observed also in the present work. It was reported that the protease chymotrypsin, also identified in this work for DP, is present in the infection of

locust (Fang et al., 2009) and other insects (Freimoser et al., 2005) by *M. anisopliae*, as well as carboxypeptidases produced during infection process of several fungi (Freimoser et al., 2003; Kanauchi and Bamforth, 2001; Zaugg et al., 2008). Based on this observation, given the high proteolytic activities identified in *M. anisopliae* strain E6, it is tempting to consider that each protease could have different biological properties and functions which are manifested at different infection stages.

Chitinases are hydrolytic enzymes secreted by many entomo-, and mycopathogenic fungi (Fang et al., 2005; Ulhoa and Peberdy, 1991). For *M. anisopliae*, due to cuticle composition, chitinases and proteases work synergistically to degrade host cuticles during the penetration process (Pedrini et al., 2007; Silva et al., 2005; St Leger et al., 1986a, 1996b). Chitinases identified in this work were detected for all antisera tested. Although both chitinases present same molecular mass, the pI is different, which indicate secretion of different isoforms. Other reports identified and detected chitinases in media mimicking host-infection (Krieger de Moraes et al., 2003; St Leger et al., 1996b).

DNase activity was detected during *M. anisopliae* growth on *M. sexta* cuticle (St Leger et al., 1986b), and in microarray, the gene that codifies this protein was upregulated in growth media containing beetle and gypsy moth cuticles (Freimoser et al., 2005). For *Tritrichomonas foetus*, a protozoan that parasitizes cows, Dnase plays a role in pathogenicity and adhesion (Greenwell et al., 2008). According to our results, the *M. anisopliae* DNase is important in the infection of *R. microplus* and *D. peruvianus*, but probably not in the early stages of the infection process. Interestingly, this spot was not stained with coomassie blue, but it was detected with antiserum and the protein identified, showing the efficiency of the immunoproteomic technique. A proline-rich

protein was also found in DP cuticle. Proteins containing a motif rich in proline perform several functions, including protein-protein interactions and stress response (He et al., 2002), and play a role as co-regulators of pathogenicity, as previously reported for other proteins that do not directly act in infection (Freimoser et al., 2005).

Our results showed that *M. anisopliae* protein secretion response varies depending on the different carbon sources used for induction, suggesting that *M. anisopliae* is able to modify the production of these proteins depending on the environment and/or host. In fact, this ability has been supported when comparing spontaneous *M. anisopliae pr1*A- and *pr1*B-deficient mutants. In deficient mutants infectivity decreases for *Tenebrio molitor*, but was the same when compared to wild type for *Galleria mellonella* (Wang et al., 2002). Also, the microarray analysis revealed differences in gene regulation, indicating that *M. anisopliae* can respond in a precise and specialized way to specific hosts (Freimoser et al., 2005).

The demonstration of the differential secretion of proteins against arthropod indicates the ability of *M. anisopliae* to direct the production of these proteins to different infection stages and hosts. This research represents a significant contribution to the understanding of the mechanisms of adaptation to hosts and the factors involved. In conclusion, we could verify the specificity of proteases: Pr1I, chymotrypsin and trypsin seems to be present only in *D. peruvianus*, which supports the idea that probably the differences in hosts and respective cuticles induce the secretion of specific pathogenicity factors and the synergistic effect they cause. Also, the identification of Pr1I, Pr1J and DNase 1 proteins suggests a novel aspect: their participation in the *M. anisopliae* infection process. The identification of proteins present also in SSP, compared with

secreted proteins against hosts, allowed the identification of factors that may be involved also in the early stages of infection. Future studies with the factors herein identified are very important to characterize their real roles in infection and pathogenicity, and consequently lead to a better understanding of the whole infection process.

Acknowledgements

We thank Dr. Jay W. Fox and Dr. Nicholas E. Sherman from the Microbiology Department, University of Virginia for their assistance with the mass spectrometric analyses. This work was supported by grants and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Bagga, S., Hu, G., Screen S.E., St Leger, R.J., 2004. Reconstruction the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae*. Gene 324, 159-169.

Barbey, C., Budin-Verneuil, A., Cauchard, S., Hartke, A., Laugier, C., Pichereau, V., Petry, S., 2008. Proteomic analysis and immunogenicity of secreted proteins from *Rhodococcus equi* ATCC 33701. Vet. Microbiol. in press.

Bogo, M.R., Rota, C.A., Pinto Jr, H., Ocampos, M., Correa, C.T., Vainstein, M.H., Schrank, A., 1998. A chitinase encoding gene (chit1 gene) from the

entomopathogenic *Metarhizium anisopliae*: isolation and characterization of genomic and full-length cDNA. Curr. Microbiol. 37, 221-225.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye biding, Anal. Biochem. 72, 248-254.

Bye, N.J., Charnley, A.K., 2008. Regulation of cuticle-degrading subtilisin proteases from the entomopathogenic fungi, *Lecanicillium* spp: implications for host specificity. Arch. Microbiol. 189, 81-92.

Cove, D.J., 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochim Biophys Acta. 113, 51-56.

Coumans, J.V., Poljak, A., Raftery, M.J., Backhouse, D., Pereg-Gerk, L., 2009. Analysis of cotton (*Gossypium hirsutum*) root proteomes during a compatible interaction with the black root rot fungus *Thielaviopsis basicola*. Proteomics 9, 335-349.

Dutra, V., Nakazato, L., Broetto, L., Schrank, I.S., Henning Vainstein, M.H., Schrank, A., 2004. Application of representational difference analysis to identify sequence tags expressed by *Metarhizium anisopliae* during the infection process of the tick *Boophilus microplus* cuticle. Res. Microbiol. 155, 245-251.

Fang, W., Leng, B., Xiao, Y., Jin, K., Ma, J., Fan, Y., Feng, J., Yang, X., Zhang, Y., Pei, P., 2005. Cloning of *Beauveria bassiana* chitinase gene *Bchit1* and its application to improve fungal strain virulence. Appl. Environ. Microbiol. 71, 363-370.

Fang, W., Pava-Ripoll, M., Wang, S., St Leger, R.J., 2009. Protein kinase A regulates production of virulence determinants by the entomopathogenic fungus, *Metarhizium anisopliae*. Fung. Genet. Biol. 46, 277-285.

Frazzon, A.P.G., Da Silva Vaz Junior, I., Masuda, A., Schrank, A., Vainstein, M.H., 2000. *In vitro* assessment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus*. Vet. Parasitol. 94, 117-125.

Freimoser, F.M., Screen, S., Bagga, S., Hu, G., St Leger, R.J., 2003. Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. Microbiol. 149, 239-247.

Freimoser, F.M., Hu, G., St Leger, R.J., 2005. Variation in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation *in vitro*. Microbiol. 151, 361-371.

Gillespie, J.P., Bateman, R., Charnley, A.K., 1998. Role of cuticle-degrading proteases in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria.* J. Invert. Pathol. 71, 128-137.

Greenwell, P., Younes, M., Rughooputh, S., 2008. Purification and analysis of DNases of *Tritrichomonas foetus*: Evidence that these enzymes are glycoproteins. Int. J. Parasitol. 38, 749-756.

He, C.Y., Zhang, J.S., Chen, S.Y., 2002. A soybean gene encoding a proline-rich protein is regulated by salicylic acid, an endogenous circadian rhythm and by various stresses. Theor. Appl. Genet. 104, 1125-1131.

Kaaya, G.P., Munyini, D.M., 1995. Biocontrol potential of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* for tsetse flies (*Glossina* spp.) at developmental sites. J. Invertebr. Pathol. 66, 237-241.

Kanauchi, M., Bamforth, C.W., 2001. Growth of *Trichoderma viride* on crude cell wall preparations from barley. J. Agric. Food Chem. 49, 883-887.

Kershaw, M.J., Moorhouse, E.R., Bateman, R., Reynolds, S.E., Charnley, A.K., 1999. The role of dextruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. J. Invertebr. Pathol. 74, 213- 223.

Krieger de Moraes, C., Schrank, A., Vainstein, M.H., 2003. Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. Curr. Microbiol. 46, 205-210.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lazzarini, G.M.J., Rocha, L.F.N., Luz, C., 2006. Impact of moisture on in vitro germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma infestans*. Mycol. Res. 110, 485-492.

Lord, J.C., Howard, R.W., 2004. A proposed role for the cuticular fatty amides of *Liposcelis bostrychophila* (Psocoptera: Liposcelidae) in preventing adhesion of entomopathogenic fungi with dry-conidia. Mycopathol. 158, 211-217.

Murad, A.M., Noronha, E.F., Miller, R.N.G., Costa, F.T., Pereira, C.D., Mehta, A., Caldas, R.A., Franco, O.L., 2008. Proteomic analysis of *Metarhizium anisopliae* secretion in the presence of the insect pest *Callosobruchus maculates*. Microbiology 154, 3766-3774.

Pedersen, S.K., Sloane, A.J., Prasad, S.S., Sebastian, L.T., Lindner, R.A., Hsu, M., Robinson, M., Bye, P.T., Weingerger, R.P., Harry, J.L., 2005. An immunoproteomic approach for identification of clinical biomarkers for monitoring disease: application to cystic fibrosis. Mol. Cell Proteomics 4, 1052-1060.

Pedrini, N., Crespo, R., Juárez, M.P., 2007. Biochemistry of insect epicuticle degradation by entomopathogenic fungi. Comp Biochem Physiol C Toxicol Pharmacol. 146, 124-37.

Quesada-Moraga, E., Santos-Quirós, R., Valverde-García, P., Santiago-Álvarez, C., 2004. Virulence, horizontal transmission, and sub lethal reproductive effects of *Metarhizium anisopliae* (Anamorphic fungi) on the German cockroach (Blattodea: Blattellidae). J. Invertebr. Pathol. 87, 51-58.

Scholte, E.J., Takken, W., Knols, B.G.J., 2007. Infection of adult *Aedes aegypti* and *Ae. albopictus* mosquitoes with the entomopathogenic fungus *Metarhizium anisopliae*. Acta Trop. 102, 151-158.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. 68, 850-858.

Shin, G.W., Palaksha, K.J., Kim, Y.R., Nho, S.W., Cho, J.H., Heo, N.E., Heo, G.J., Park, S.C., Jung, T.S., 2007. Immunoproteomic analysis of capsulate and non-capsulate strains of *Lactococcus garvieae*. Vet. Microbiol. 119, 205-212.

Silva, W.O.B., Mitidieri, S., Schrank, A., Vainstein, M.H., 2005. Production and extraction of an extracellular lipase from the entomopathogenic fungus *Metarhizium anisopliae*. Proc. Biochem. 40, 321-326.

Silva, M.V., Santi, L., Staats, C.C., Costa, A.M., Colodel, E.M., Driemeier, D., Vainstein, A., Schrank, A., 2005. Cuticle-induced endo/exoacting chitinase CHIT30 from *Metarhizium anisopliae* is encoded by an ortholog of the *chi3* gene. Res. Microbiol. 156, 382-392.

St Leger, R.J., Charnley, A.K., Cooper, R.M., 1986a. Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. J. Invertebr. Pathol. 48, 85-95.

St Leger, R.J., Butt, T.M., Staples, R.C., Roberts, D.W., 1989. Synthesis of proteins including a cuticle-degrading protease during differentiation of the entomopathogenic fungus *Metarhizium anisopliae*. Exp. Mycol. 13, 253-262.

St Leger, R.J., Joshi, L., Bidochka, R.J., Rizzo, N.W., Roberts, D.W., 1996a. Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Appl. Env. Microbiol. 62, 1257-1264.

St Leger, R.J., Joshi, L., Bidochka, M.J., Rizzo, N.W., Roberts, D.W., 1996b. Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae, M. flavoviride,* and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Appl. Environ. Microbiol. 62, 907-912.

St Leger, R.J., Joshi, L., Bidochka, M.J., Roberts, D.W., 1996c. Construction of an improved mycoinsecticide overexpressing a toxic protease. Proc. Natl. Acad. Sci. USA 93, 6349-6354.

Suarez M.B., Sanz, L., Chamorro M.L., Rey M., González F.J., Llobell A., Monte E., 2005. Proteomic analysis of secreted proteins from *Trichoerma harzianum*: identification of a fungal cell wall-induced aspartic protease. Fungal Genet. Biol. 42, 924-934.

Ulhoa, C.J., Peberdy, J.F., 1991. Regulation of chitinase synthesis in *Trichoderma harzianum*. J. Gen. Microbiol. 137, 2163-2169.

Wang, C., Typas, M.A., Butt, T.M., 2002. Detection and characterization of *pr*1 virulent gene deficiencies in the insect pathogenic fungus *Metarhizium anisopliae*. FEMS Microbiol. Lett. 213, 251-255.

Wang, C., St Leger, RJ., 2005. Developmental and transcriptional responses to host and nonhost cuticles by the specific locust pathogen *Metarhizium anisopliae* var. *acridum*. Eukaryot. Cell 4, 937-947.

Wang, C., Hu, G., St Leger, R.J., 2005. Differential gene expression by *Metarhizium anisopliae* growing in root exudates and host (*Manduca sexta*) cuticle or hemolymph reveals mechanisms of physiological adaptation. Fung. Genet. Biol. 42, 704-718.

Wang, Y., Yang, L., Xu, H., Li, Q., Ma, Z., Chu, C., 2005. Differential proteomic analysis of proteins in wheat siles induced by *Fusarium graminearum*. Proteomics 5, 4496-4503.

Wu, Z., Zhang, W., Lu, C., 2008. Immonoproteomic assay of surface proteins of *Streptococcus suis* serotype 9. FEMS Immunol. Med. Microbiol. 53, 52-59.

Xu, J., Baldwin, D., Kindrachuk, C., Hegedus, D.D., 2006. Serine proteases and metalloproteases associated with pathogenesis but not host specificity in the Entomophthoralean fungus *Zoophthora radicans*. Can. J. Microbiol. 52, 550-559.

Zaugg, C., Jousson, O., Léchenne, B., Staib, P., Monod, M., 2008. *Trichophyton rubrum* secreted and membrane-associated carboxypeptidases. Int. J. Med. Micr. 298, 669-682.

Table 1 Results of spot identification of *M. anisopliae in vitro* infection conditions using immunoproteomic technique. The supernatant of *M. anisopliae* cultured in *D. peruvianus* cuticle (DP) was used as antigenic proteins, and spots were immunodetect using antiserum anti-*D. peruvianus* (DP) cuticle, anti-*R. microplus* cuticle (RM) and anti-spore surface proteins (SSP).

Spot no.	RM	DP	SSP	Identification	Accession no.	Theoretical pI/mass (kDa)	Observed pI/mass (kDa)	Sequences (unic peptides)
1		X		Chymotrypsin (*M. anisopliae*)	gi|5042248	5.07/38279	5.2/66	KGATVNYPQGAVSGLTRT
2		X		Chymotrypsin (*M. anisopliae*)	gi|5042248	5.07/38279	5.2/64	KGATVNYPQGAVSGLTRT RSGSTTQVHCGTIGAKG
3		X		Subtilisin-like protease Pr1I (*M. anisopliae* var. *anisopliae*)	gi|6634475	6.25/40338	5.6/40	KGVVANM*SLGGGYSAAINQAAAKM
4		X		Subtilisin-like protease Pr1I (*M. anisopliae* var. *anisopliae*)	gi|6634475	6.25/40338	5.8/40	KGVVANM*SLGGGYSAAINQAAAKM KVLSDQGSGDYSGILAGM*DFAIQDSRT
5		X	X	Subtilisin-like protease Pr1I	gi|6634475	6.25/40338	6.0/40	KGVVANM*SLGGGYSAAINQAAAKM

				(*M. anisopliae* var. *anisopliae*)				KVLSDQGSGDYSGILAGM*DFAIQDSRT
6	X		X	Subtilisin-like Pr1J (*M. anisopliae*)	gi|16215660	6.09/42279	5.6/42	KDATDEDVQGHGTWVAGIVGSKT KATAVKDGTVNLVAYNGII KTGSGTSQAAPHVAGLAAYLAVAKN
7	X		X	Subtilisin-like Pr1J (*M. anisopliae*)	gi|16215660	6.09/42279	5.8/42	KATAVKDGTVNLVAYNGII
6	X	X		Carboxypeptidase (*M. anisopliae*)	gi|197253670	6.59/45953	9.8/45	RGASAGNSPEVKS RTPNNAALQALAKG
9	X		X	Elastase-like serine protease (*M. anisopliae* var. *anisopliae*)	gi|16506140	8.27/37340	6.6/20	RSFISGQETDGHGHGTHCAGTIGSKS RDAQNTSPASEPSACTVGATDSSDRRS
10		X		Elastase-like serine protease (*M. anisopliae* var. *anisopliae*)	gi|16506140	8.27/37340	4.6/42	KVLDNQGSGSYSGIISGM*DYVASDSKT
11	X	X		DNase1 protein (*M. anisopliae*)	gi|6624948	4.94/17736	4.5/20	KQM*YPASGKS RTIYFTPNPGFPEVAPVTCNNKQKT KSPM*SGCPVFPCNNAYYLPDDVQTKV

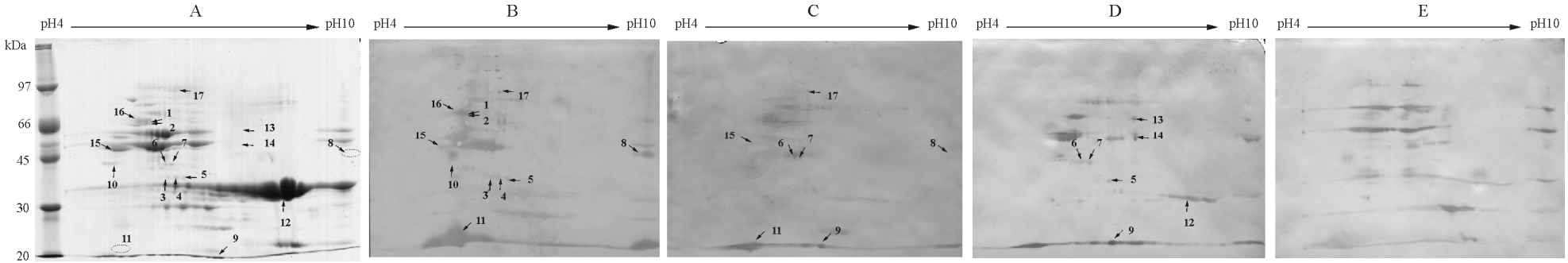
								KTVVNFPDQWIGNYYAVQKG
12			X	Carboxypeptidase (*M. anisopliae*)	gi|197253670	6.59/45953	8.4/32	KADNVFTIELRD KAVVDKYDFYM*FPIVNVDGFKY KDIASQYPSNVKS RGASAGNSPEVKS RNQGSSCLGTDPNRN RSVHGTTFAYGPVCNVIYQVAGGSIDWVQDVLKA KSYIAFLDKI RTPNNAALQALAKG KWDGPGSSTNPCTETYRG KYATDSAVKA
13			X	Trypsin-related protease (*M. anisopliae*)	gi|4768909	5.68/26289	7.0/60	RLQEGGATPSQLQKV
14			X	Chitinase (*M. anisopliae* var. *acridum*)	gi|71494532	6.22/46129	7.0/46	KIVLGM*PIYGKS RKGPDSLIGTSSNKL RTQELISYDTPDITKE KLGGPDATENLLNYPDSKY
15	X	X		Chitinase (*M. anisopliae* var.	gi|71494532	6.22/46129	4.5/46	KIVLGM*PIYGKS

			acridum)				KLGGPDATENLLNYPDSKY
							RKGPDSLIGTSSNKL
							RTQELISYDTPDITKE
			Chitinase (*M. anisopliae*)	gi|63098857	5.38/46181		KLHLGDLGKV
							KFAQGYHFQLSIAAPAGPANYNKL
16		X	Proline-rich protein 5MeD	gi|2352910	4.99/55702	5.0/66	KATLVNSNVNAGPSGSHINAIGFNIRD
			(*M. anisopliae*)				RDTGSGSNELYEVDLASGKT
							KGPASSGNDGAGCGGPLAPLPPTGPLSPLKC
							KVADSTAVEFIAKG
							RVKVADSTAPVEFIAKG
							RWDM*STHIFTVIRD
17	X	X	Subtilisin-like serine protease	gi|38640823	7.14/40303	6.0/97	RDAQNTSPASEPTACTVGATDSSDRRS
			Pr1A (*M. anisopliae* var.				
			anisopliae)				

[a] pI and MW theorethical calcutated according ExPASY.
M* methionine oxidation

Legend figures:

Fig. 1. 2DE map and immunoblot of secreted proteins of *M. anisopliae* cultured in minimal medium plus *D. peruvianus* cuticle, with the immunoreactive proteins indicated by spot number. (A) 2DE gel stained with coomassie blue R250. Other gels were run in parallel and were used for immunoblotting using (B) anti-*D. peruvianus* (anti-DP) (1:1000), (C) anti-*R. microplus* (anti-RM) (1:2000), (D) anti-spore surface proteins (anti-SSP) (1:1000) or (E) anti-glucose (anti-G) (1:1000) sera. Spots identified by MS/MS are labeled and identified in Table 1.



5. CONCLUSÕES

Fungos entomopatogênicos envolvem estratégias distintas para colonizar seus hospedeiros. *M. anisopliae* produz enzimas extracelulares que degradam componentes protéicos, lipídicos e quitinosos presentes na cutícula dos artrópodes, permitindo a penetração da hifa, levando ao parasitismo. *M. anisopliae* é amplamente utilizado para o controle biológico de pestes da agricultura, pecuária e saúde pública, sendo um dos agentes de biocontrole mais utilizados e estudados.

Para que um microrganismo seja utilizado para o controle biológico a campo, algumas características devem ser levadas em consideração, tais como: não toxicidade, especificidade, fácil manuseio, produção de propágulos infecciosos em substratos simples e de baixo custo e estabilidade sob condições ambientais. A utilização de arroz para a produção de esporos de *M. anisopliae* mostrou-se eficiente, sendo um substrato de baixo custo, gerando uma alta produtividade de esporos e favorecendo a resistência a radiação UV, característica importante para a persistência do esporo a campo. Porém, lavagens sucessivas podem causar a degradação do arroz, observada pela aglomeração dos grãos após a segunda lavagem efetuada, o que dificultou o desenvolvimento do fungo e, consequentemente, a esporulação.

Outra característica importante para a utilização de microrganismos a campo é o tipo de formulação utilizada. Formulações contendo óleos vegetais, quando comparadas a formulações aquosas, favorecem a infecção de fungos pelo aumento da persistência do microrganismo e proteção contra fatores adversos. Aqui, a utilização de 10% óleo de soja à suspensão de esporos de *M. anisopliae* foi três vezes mais efetiva para o controle de *D. peruvianus*, comparada à suspensão aquosa. Interessantemente, as ninfas apresentaram-se mais suscetíveis a *M. anisopliae* em relação aos adultos, o que sugere diferenças no

sistema imune e na composição de hidrocarbonetos epicuticulares entre ninfas e adultos. Estudos de microscopia eletrônica de varredura (MEV) evidenciaram a penetração direta de *M. anisopliae* pela cutícula do inseto, bem como a seqüência de eventos caracterizada para fungos entomopatogênicos. Para *D. peruvianus*, as articulações foram consideradas pontos preferenciais de penetração.

Para o conídio, unidade de infecção de fungos, a adesão à superfície do hospedeiro é o evento inicial para o processo de patogenicidade. Utilizando uma metodologia específica para extração de proteínas presentes na superfície do esporo, foram identificadas proteases (incluindo Pr1 e Pr2), quitinases e lipases, descritas como possíveis fatores de patogenicidade por degradarem compostos da cutícula de artrópodes. Além disso, foram detectadas atividades de fosfolipase C – identificada pela primeira vez em esporos - trealase e enzimas envolvidas na proteção contra espécies reativas de oxigênio. A multiplicidade de enzimas detectadas na superfície do esporo de *M. anisopliae* reflete a habilidade do fungo em responder e "atacar" o hospedeiro durante a patogênese. O arsenal enzimático compreende atividades relacionadas com processos fisiológicos e ecológicos, que são vitais para a sobrevivência do fungo e adaptação a diversos habitats, como saprófita e entomoparasita.

Em comparação com atividades enzimáticas e purificação de proteínas já descritas para *M. anisopliae*, pouco se sabe sobre suas funções diretamente no sistema infectivo e no biocontrole. Estruturas da superfície, topografia e composição química da cutícula dos hospedeiros afetam a adesão de esporos fúngicos e induzem a secreção de proteínas específicas. Utilizando a metodologia de imunoproteômica diferencial, foram observadas diferenças na secreção de proteínas de *M. anisopliae* em relação aos dois hospedeiros testados: *D. peruvianus* e *R. microplus*. Uma proteína transportadora de ácido carboxílico foi identificada somente na condição de infecção do carrapato *R. microplus*, enquanto

quimotripsina e Pr1I foram identificadas na condição de infecção do percevejo *D. peruvianus*. Outras proteínas, como Pr1A, quitinases, proteína rica em prolina, carboxipeptidase, Pr1J, entre outras, foram identificadas para os dois hospedeiros. Estes resultados indicam que a secreção de proteínas de *M. anisopliae* varia dependendo da cutícula utilizada, sugerindo que *M. anisopliae* é capaz de modificar a produção destas proteínas dependendo do ambiente e/ou hospedeiro.

Além disso, proteínas que não são detectadas por corantes usualmente utilizados, foram identificadas através do reconhecimento de anticorpos, tornando a imunoproteômica uma ferramenta interessante para análise de proteínas pouco abundantes presentes nas amostras.

Estas descobertas reforçam a participação de proteases e quitinases no processo de infecção e provêm fontes adicionais para o estudo da patogenicidade, especialmente na fase inicial, com a adesão do esporo e a secreção de proteínas envolvidas neste processo e na especificidade dos hospedeiros. Proteínas com função pouco conhecida para *M. anisopliae* devem agora ser mais bem analisadas, bem como o estudo de quais fatores afetam a secreção de enzimas descritas como fatores de patogenicidade em hospedeiros específicos. Além disso, o entendimento da função das enzimas presentes no esporo auxiliará o entendimento dos mecanismos envolvidos no primeiro estágio da infecção.

6. PERSPECTIVAS

Incrementar pesquisas futuras que possibilitem avaliar o efeito sinergístico entre a formulação de *M. anisopliae* contendo 10% de óleo de soja e uma dieta contendo proteínas do tipo canatoxina e peptídeos derivados para o controle de *D. peruvianus*, a fim de aumentar a eficiência do biocontole.

Estudar as proteínas de *M. anisopliae* identificadas neste trabalho por imunoproteômica e detectadas na superfície do esporo, a fim de avaliar sua participação na infecção e especificidade de diferentes hospedeiros artrópodes.

7. REFERÊNCIAS BIBLIOGRÁFICAS

ALVES SB. (1998) Controle microbiano de insetos. FEALQ, Piracicaba, 1163p.

ALVES LFA, FILHO AB. (1998). Formulação de entomopatógenos: uma boa formulação é a base para o sucesso de um inseticida microbiano. Biotecnologia Ciência e Desenvolvimento, 5: 32-34.

AKBAR W, LORD JC, NECHOLS JR, HOWARD RW. (2004). Diatomaceous earth increases the efficacy of *Beauveria bassiana* against *Tribolium castaneum* larvae and increases conidia attachment. Journal of Economic Entomology, 97: 273-280.

ANGUS BM. (1996). The history of the cattle tick *Boophilus microplus* in Australia and achievements in its control. International Journal of Parasitology, 26: 1341-1355.

ARENKOV P, KUKHTIN A, GEMMELL A, VOLOSHCHUK S, CHUPEEVA V, MIRZABEKOV A. (2000). Protein microchips: use for immunoassay and enzymatic reactions. Analytical Biochemistry, 278: 123-131.

ARRUDA W. (2005). Caracterização molecular e morfofisiológica de diferentes isolados do fungo entomopatogênico *Metarhizium anisopliae* e análise morfológica do processo de infecção em *Boophilus microplus*. Tese de Doutorado. Curso de Pós-graduação em Biologia Celular e Molecular (PPGBCM) da Universidade Federal do Rio Grande do Sul, Porto Alegre.

ARRUDA W, LUBECK I, SCHRANK A, VAINSTEIN MH. (2005). Morphological alterations of *Metarhizium anisopliae* structures during the penetration events leading to the infection of the cattle tick *Boophilus microplus.* Experimental and Applied Acarology, 37: 231-244.

ASIF AR, OELLERICH M, AMSTRONG VW, RIEMENSCHNEIDER B, MONOD M, REICHARD U. (2006). Proteome of conidial surface associated proteins of

Aspergillus fumigatus reflecting potential vaccine candidates and allergens. Journal of Proteome Research, 5: 954-962.

BAGGA S, HU G, SCREEN SE, ST LEGER RJ. (2004). Reconstructing the diversification of subtilisins in the entomopathogenic fungus *Metarhizium anisopliae*. Gene, 324: 159-169.

BARATTO CM, DUTRA V, BOLDO JT, LEIRIA LB, VAINSTEIN MH, SCHRANK A. (2006). Isolation, characterization and transcriptional analysis of the chitinase chi2 gene (DQ011663) from the biocontrol fungus *Metarhizium anisopliae* var. *anisopliae*. Current Microbiology, 53: 217-221.

BARBEY C, BUDIN-VERNEUIL A, CAUCHARD S, HARTKE A, LAUGIER C, PICHEREAU V, PETRY S. (2008). Proteomic analysis and immunogenicity of secreted proteins from *Rhodococcus equi* ATCC 33701. Veterinary Microbiology, in press.

BARNES DA, MONIN A, HUANG JX, GOUSSET L, WU J, GUT J, DOYLE P, DUBREMETZ JF, WARD H, PETERSEN C. (1998). A novel multi-domain mucin-like glycoprotein of *Cryptosporidium parvum* mediates invasion. Molecular Biochemistry and Parasitology, 96: 93-110.

BATTA YA. (2003). Production and testing of novel formulations of the entomopathogenic fungus *Metarhizium anisopliae* (Metschinkoff) Sorokin (Deuteromycotina: Hyphomycetes). Crop Protection, 22: 415-422.

BIDOCHKA MJ, KHACHATOURIANS GG. (1990). Identification of *Beauveria bassiana* extracellular protease as a virulence factor in pathogenicity toward the migratory grasshopper, *Melanoplus sanguimipes*. Journal of Invertebrate Pathology, 56: 362-370.

BIDOCHKA MJ, ST LEGER RJ, ROBERTS DW. (1997). Mechanisms of deuteromycete fungal interactions in grasshoppers and locusts: an overview. Memoirs of the Entomology Society of Canada, 171: 231-224.

BIEMANN K. (1963). Mass spectrometry. Annual Review of Biochemistry, 32: 755-780.

BIO CONTROLE (2009). www.biocontrole.com.br/armadilhas (28 de março).

BITTENCOURT SET, CASTRO LA, FARIAS SE, BAO SN, SCHRANK A, VAINSTEIN MH. (2004). Purification and ultrastructural localization of a copper–zinc superoxide dismutase (CuZnSOD) from the entomopathogenic and acaricide fungus *Metarhizium anisopliae*. Research in Microbiology, 155: 681-687.

BJELLQVIST B, EK K, RIGHETTI PG, GIANAZZA E, GORG A, WESTERMEIER R, POSTEL W. (1982). Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. Journal of Biochemistry and Biophysic Methods, 6: 317-339.

BLOMQUIST GJ, NELSON DR, de RENOBALES M. (1987). Chemistry, biochemistry and physiology of insect cuticular lipids. Archives of Insect Biochemistry and Physiology, 6: 227-265.

BLUHM BH, DHILLON B, LINDQUIST EA, KEMA GH, GOODWIN SB, DUNKLE LD. (2008). Analyses of expressed sequence tag from the maize foliar pathogen *Cercospora zea-maydis* identify novel genes expressed during vegetative infections, and reproductive growth. BMC Genomics, 9: 523-540.

BOUCIAS DG, LATGE JP. (1988). Non-specific induction of germination of *Coridiobolus obscurus* and *Nomuraea rileyi* host and non-host cuticle extracts. Journal of Invertebrate Pathology, 51: 168-171.

BRISOLLA AD, BERGMANN EC, IMENES SDL. (1992). Aspectos biológicos de *Dysdercus peruvianus* Guérin-Menéville, 1831, em condições de laboratório. Arquivos do Instituto Biológico. São Paulo, 59: 19-22.

CABI BIOSCIENCE (1999). CABI official noted. FMI Silwood Park, Ascot, Berks, UK.

CAPRIOLI RM, FARMER TB, GILE J. (1997). Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. Analytical Chemistry, 69: 4751-4760.

CARBERRY S, NEVILLE CM, KAVANAGH KA, DOYLE S. (2006). Analysis of major intracellular proteins of *Aspergillus fumigatus* by MALDI mass spectrometry: identification and characterization of an elongation factor 1B protein with glutathione transferase activity. Biochemical and Biophysical Research Communications, 341: 1096-1104.

CARSOLIO C, BENHAMOU N, HARAN S, CORTES C, GUTIERREZ A, CHET I, HERRERA-ESTRELLA A. (1999). Role of the *Trichoderma harzianum* endochitinase gene, *ech42*, in mycoparasitism. Applied and Environmental Microbiology, 65: 929-935.

CHAURAND P, SANDERS ME, JENSEN RA, CAPRIOLI RM. (2004). Proteomics in diagnostic pathology: profiling and imaging proteins directly in tissue sections. American Journal of Pathology, 165: 1057-1068.

CLARKSON JM, CHARNLEY AK. (1996). New insights into the mechanisms of fungl pathogenesis in insects. Trends in Microbiology, 4: 197-203.

COLE SCJ, CHARNLEY AK, COOPER RM. (1993). Purification and partial characterisation of a novel trypsin-like cysteine proteinase from *Metarhizium anisopliae*. FEMS Microbiology Letters, 113: 189-196.

CORREIA ACB, FIORIN AC, MONTEIRO AC, VERISSIMO CJ. (1998). Effects of *Metarhizium anisopliae* on the tick *Boophilus microplus* (Acari: Ixodidae) in stabled cattle. Journal of Invertebrate Pathology, 71: 189-191.

COUMANS JV, POLJAK A, RAFTERY MJ, BACKHOUSE D, PEREG-GERK L. (2009). Analysis of cotton (*Gossypium hirsutum*) root proteomes during a compatible interaction with the black root rot fungus *Thielaviopsis basicola*. Proteomics, 9: 335-349.

DAVÉ A, JEENES DJ, MACKENZIE DA, ARCHER DB. (2006). HacA-independet induction of chaperone-encoding gene *bipA* in *Aspergillus niger* strains overproducing membrane proteins. Applied and Environmental Microbiology, 72: 953-955.

DE LA CRUZ J, HIDALGO-GALLEGO A, MLORA J, BENITEZ T, PINTOR-TORO JA, LLOBELL A. (1992). Isolation and characterization of three chitinases from *Trichoderma harzianum*. European Journal of Biochemistry, 206: 859-867.

DONG C, ZHANG J, HUANG H, CHEN W, HU Y. (2007). Pathogenicity of a new China variety of *Metarhizium anisopliae* (*M. anisopliae* var. *dcjhyium*) to subterranean termite *Odontotermes formosanus*. Microbiological Research, 162: 53-61.

DRIVER F, MILNER RJ, TRUEMAN JWH. (2000). A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. Mycological Research, 104: 134-150.

DUTRA V, NAKAZATO L, BROETTO L, SCHRANK IS, VAINSTEIN MH, SCHRANK A. (2004). Application of representational difference analysis to identify sequence tags expressed by *Metarhizium anisopliae* during the infection process of the tick *Boophilus microplus* cuticle. Research in Microbiology, 155: 245-251.

EDMAN P. (1949). A method for the determination of amino acid sequence of peptides. Archives of Biochemistry and Biophysics, 22: 475-483.

ESTRADA-PEÑA A, GARCÍA Z, SÁNCHEZ HF. (2006). The distribution and ecological preferences of *Boophilus microplus* (Acari: Ixodidae) in Mexico. Experimental and Applied Acarology, 38: 307-316.

EUBEL H, MEYER EH, TAYLOR NL, BUSSEL JD, OTOOLE N, HEAZLEWOOD JL, CASLEDEN I, SMALL ID, SMITH SM, MILLAR AH. (2008). Novel proteins, putative membrane transporters and an integrated metabolic network are revealed by quantitative proteomic analysis of *Arabidopsis* cell culture peroxisomes. Plant Physiology, 148: 1809-1829.

EVANS DE, MARTINS JR, GUGLIELMONE AA. (2000). A review of the ticks (Acari: Ixodidae) of Brazil, their hosts and geographic distribution: the state of Rio Grande do Sul, southern Brazil. Memórias do Instituto Oswaldo Cruz, 95: 453-470.

FANG W, LENG B, XIAO Y, JIN K, MA J, FAN Y, FENG J, YANG X, ZHANG Y, PEI P. (2005). Cloning of *Beauveria bassiana* chitinase gene *Bchit1* and its application to improve fungal strain virulence. Applied and Environmental Microbiology, 71: 363-370.

FANG W, PEI Y, BIDOCHKA MJ. (2007). A regulator of a G protein signaling (RGS) gene, cag8, from the insect-pathogenic fungus *Metarhizium anisopliae* is involved in conidiation, virulence and hydrophobin synthesis. Microbiology, 153: 1017-1025.

FARIA MR, WRAIGHT SP. (2007). Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biological Control, 43: 237-256.

FERRON P. (1985). Fungal control. In: KERKUT GA, GILBERT LI (Eds.) Comprehensive insect physiology, biochemistry and pharmacology. Academic Press, New York. P. 313-346.

FRAZZON APG, VAZ JUNIOR I, MASUDA A, SCHRANK A, VAINSTEIN MH. (2000). *In vitro* assessment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus*. Veterinary Parasitology, 94: 117-125.

FREIMOSER FM, SCREEN S, BAGGA S, HU G, ST LEGER RJ. (2003). Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. Microbiology, 149: 239-247.

FREIMOSER FM, HU G, ST LEGER RJ. (2005). Variation in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation *in vitro.* Microbiology, 151: 361–371.

FURLONG J, CHAGAS ACS, NASCIMENTO CB. (2002). Comportamento e ecologia de larvas do carrapato *B. microplus* em pastagem de *Brachiaria decumbens*. Brazilian Journal Veterinary Research Animal Science, 39: 213-217.

GENG H, ZHU L, YUAN Y, ZHANG W, LI W, WANG J, ZHENG Y, WEI K, CAO W, WANG H, JIANG Y. (2008). Identification and characterization of novel immunogenic proteins of *Streptococcus suis* serotype 2. Journal of Proteome Research, **7**: 4132-4142.

GILLESPIE JP, BATEMAN R, CHARNLEY AK. (1998). Role of cuticle-degrading proteases in the virulence of *Metarhizium* spp. for the desert locust, *Schistocerca gregaria*. Journal of Invertebrate Pathology, 71: 128-137.

GIMÉNEZ-PECCI MP, BOGO MR, SANTI L, MORAES CK, CORRÊA CT, VAINSTEIN MH, SCHRANK A. (2002). Characterization of mycoviruses and

analyses of chitinases secretion in the biocontrol fungus *Metarhizium anisopliae*. Current Microbiology, 45: 334-339.

GOETTEL MS, HAJEK AE. (2000). Evaluation of non-target effects of pathogens used for management of arthropods. In: WAJNBERG E, SCOTT JK, QUIMBY PC (Eds) Evaluating indirect ecological effects of biological control. CAB International, Wallingford, p. 81-97.

GONÇALVES L. (2000). Biologia e comportamento de *Dysdercus ruficollis* (Linnaeus, 1764) (Hemiptera: Pyrrhocoridae) em condições de laboratório. Floresta e Ambiente, 7: 68-79.

GONZALEZ-ACUÑA D, GUGLIELMONE AA. (2005). Ticks (Acari: Ixodoidea: Argasidae: Ixodidae) of Chile. Experimental and Applied Acarology, 35: 147-163.

GOODAY GH, ZHU WY, DONNELL RW. (1992). What are the roles of chitinases in the growing fungus? FEMS Microbiology Letters, 100: 387-392.

GORG A, KLAUS A, LUCK C, WEILAND F, WEISS W. (2007). Two-dimensional electrophoresis with immobilized pH gradients for proteome analysis – A laboratory manual. http://www.wzw.tum.de/proteomik.

GRIMM C. (2001). Economic feasibility of small-scale production plant for entomopathogenic fungi in Nicaragua. Crop Protection, 20: 623-630.

GRINYER J, HUNG S, McKAY M, HERBERT BR, NEVALAINEN H. (2005). Proteromic response of the biological control fungus *Trichoderma atroviride* to growth on the cell walls of *Rhizoctonia solani*. Current Genetics, 47: 381-388.

GRISI L, MASSARD C, MOYA BORJA GE, PEREIRA JB. (2002). Impacto econômico das principais ectoparasitoses em bovinos no Brasil. A Hora Veterinária, 21: 8-10.

HASSAN A, CHARNLEY AK. (1989). Ultrastructural study of the penetration by *Metarhizium anisopliae* throug dimilin-affected cuticle of *Manduca sexta*. Journal of Invertebrate Pathology, 54: 117-124.

HOOGSTRAL H. (1985). Argasid and nuttallied ticks as parasites and vectors. Advance Parasitology, 1: 135-238.

HSIAO YM, KO JL. (2001). Determination of destruxins, cyclic peptide toxins, produced by different strains of *Metarhizium anisopliae* and their mutants induced by ethyl methane sulfonate and ultraviolet using HPLC method. Toxicon, 39: 837-841.

HU G, ST LEGER, RJ. (2004). A phylogenomic approach to reconstructing the diversification of serine proteases in fungi. Journal of Evolutionary Biology, 17: 1204-1214.

HUXHAM IM, SAMUELS KDL, HEALE JB, McCORKINDALE NJ. (1989). *In vivo* and *in vitro* assays for pathogenicity of wild-type and mutant strains of *Metarhizium anisopliae* for three insect species. Journal of Invertebrate Pathology, 53: 143-151.

ITAFORTE BioProdutos. (2009). http://www.itafortebioprodutos.com.br/. 06 de janeiro.

JACKMAN JE, GRAYHACK EJ, PHIZICKY EM. (2008). The use of *Saccharomyces cerevisiae* proteomic libraries to identify RNA-modifying proteins. Methods in Molecular Biology, 488: 383-393.

JENKINS NE. (1998). Development of mass production technology for aerial conidia for use as mycospesticides. Biocontrol News Information, 19: 21-31.

JOSHI L, ST LEGER RJ. (1999). Cloning, expression, and substrate specificity of MeCPA, a zinc carboxypeptidase that is secreted into infected tissues by the fungal entomopathogen *Metarhizium anisopliae*. Journal of Biological Chemistry, 274: 9803-9811.

JUAREZ MP, CRESPO R., FERNANDEZ GC, LEUCONA R, CAFFERATA LF. (2000) Characterization and carbon metabolism in fungi pathogenic to *Triatoma infestans*, a chagas disease vector. Journal of Invertebrate Pathology, 76: 198-207.

KAAYA GP, MUNYINI DM. (1995). Biocontrol potential of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* for tsetse flies (*Glossina* spp.) at developmental sites. Journal of Invertebrate Pathology, 66: 237-241.

KAAYA GP, HASSAN S. (2000). Entomogenous fungi as promising biopesticides for tick control. Experimental and Applied Acarology, 24: 913-926.

KERSHAW MJ, MOORHOUSE ER, BATEMAN R, REYNOLDS SE, CHARNLEY AK. (1999). The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. Journal of Invertebrate Pathology, 74: 213-223.

KOLKMAN A, DARAN-LAPUJADE P, FULLAONDO A, OLSTHOORN MMA, PRONK JT, SLIJPER M, HECK AJ. (2006). Proteome analysis of yeast response to various nutrient limitations. Molecular Systems Biology, 2:1-16.

KRIEGER DE MORAES C, SCHRANK A, VAINSTEIN MH. (2003). Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae*. Current Microbiology, 46: 205-210.

KUCERA M. (1980). Proteases from the fungus *Metarhizium anisoplaie* for *Galleria mellonella* larvae. Journal of Invertebrate Pathology, 35: 304-310.

LAVERLAM SA. (2009). http://www.laverlam.com.co. 06 de janeiro.

LAZZARINI GMJ, ROCHA LFN, LUZ C. (2006). Impact of moisture on in vitro germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma infestans*. Mycological Research, 110: 485-492.

LEAL AT, FREITAS DRJ, VAZ JR IS. (2003). Perspectivas para o controle do carrapato bovino. Acta Scientiae Veterinariae, 31: 1-11.

LIMÓN MC, PINTOR-TORO JA, BENITEZ T. (1999). Increased antifungal activity of *T.harzianum* transformants that overexpress a 33-kDa chitinase. Biological Control, 89: 254-261.

LIU ZY, LIANG ZQ, WHALLEY AJ, YAO YJ, LIU AY. (2001). *Cordyceps brittlebankisoides*, a new pathogen of grubs and its anamorph, *Metarhizium anisopliae* var. *majus*. Journal of Invertebrate Pathology, 78: 178-182.

LOMER CJ, BATEMAN RP, JOHNSON DL, LANGEWALD J, THOMAS MB. (2001) Biological control of locusts and grasshoppers. Annual Review of Entomology, 46: 667-702.

LORD JC, HOWARD RW. (2004). A proposed role for the cuticular fatty amides of *Liposcelis bostrychophila* (Psocoptera: Liposcelidae) in preventing adhesion of entomopathogenic fungi with dry-conidia. Mycopathologia, 158: 211-217.

LUBECK I, ARRUDA W, SOUZA BK, STANISÇUASKI F, CARLINI CR, SCHRANK A, VAINSTEIN MH. (2008). Evaluation of *Metarhizium anisopliae Rhipicephalus (Boophilus) microplus Dysdercus peruvianus*. Fungal Ecology 1: 78-88.

MAGALHÃES BS, MELO JA, LEITE JR, SILVA LP, PRATES MV, VINECKY F, BARBOSA EA, VERLY RM, MEHTA A, NICOLI JR, BEMQUERER MP, ANDRADE AC, BLOCH Jr C. (2008). Post-secretory events alter the peptide content of the skin secretion of *Hypsiboas raniceps*. Biochemical and Biophysical Research Comunications, 337: 1057-1061.

MAHJAN MS, TAN MW, RAHME LG, AUSUBEL FM. (1999). Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. Cell, 96: 47-56.

MARANINNO P, SANTIAGO-ÁLVAREZ C, LILLO E, QUESADA-MORAGA E. (2006) A new bioasay method reveals pathogenicity of *Metarhizium anisopliae* and

Beauveria bassiana against early stages of *Capnodis tenebrionis* (Coleoptera: Buprestidae). Journal of Invertebrate Pathology, 93: 210-213.

MATHESIUS U. (2008). Comparative proteomic studies of root-microbe interactions. Journal of Proteomics. In press.

MELO IS, AZEVEDO JL. (1998). Controle Biológico, volume I. EMBRAPA, p. 262.

MILANO P, CÔNSOLI FL, ZÉRIO NG, PARRA JRP. (1999). Ecologia, comportamento e bionomia – Exigências térmicas de *Dysdercus peruvianus* Guérin-Méneville (Heteroptera: Pyrrhocoridae), o percevejo manchador do algodão. Anais da Sociedade Entomológica do Brasil, 28: 233-238.

MILNER RJ. (2000). Current status of *Metarhizium* as a mycoinsecticide in Australia. Biocontrol News and Information, 21: 47-50.

MINI R, BERNARDINI G, SALZANO AM, RENZONE G, SCALONI A, FIGURA N, SANTUCCI A. (2006). Comparative proteomics and immunoproteomics of *Helicobacter pylori* related to different gastric pathologies. Jornal of Chromatography B: Analytical Technology and Biomedical Life Science, 833: 63: 79.

MOORE DP, BRIDGE D, HIGGINS PM, BATEMAN R, PROIR C. (1992). Ultra-violet radiation damage to *Metarhizium flavoviride* conidia and the protection given by vegetable and mineral oils and chemical sunscreens. Annals of Applied Biology 122: 605-616.

MURAD AM, LAUMANN RA, LIMA TA, SARMENTO RBC, NORONHA EF, ROCHA TL, VALADARES-INGLIS MC, FRANCO OL. (2006). Screening of entomopathogenic *Metarhizium anisopliae* isolates and proteomic analysis of secretion synthesized in response to cowpea weevil (*Callosobruchus maculatus*) exoskeleton. Comparative Biochemistry and Physiology, Part C, 142: 365-370.

MURAD AM, LAUMANN RA, MEHTA A, NORONHA EF, FRANCO OL. (2007). Screening and secretomic analysis of entomopathogenic *Beauveria bassiana* isolates in response to cowpea weevil (*Callosobruchus maculatus*) exoskeleton. Comparative Biochemistry and Physiology, Part C, 145: 333-338.

MURAD AM, NORONHA EF, MILLER RNG, COSTA FT, PEREIRA CD, MEHTA A, CALDAS RA, FRANCO OL. (2008). Proteomic analysis of *Metarhizium anisopliae* secretion in the presence of the insect pest *Callosobruchus maculatus*. Microbiology, 154: 3766-3774.

NAGEE A, ACHARYA A, SHETE A, MUKHOPADHYAYA PN, AICH BA. (2008) Molecular characterization of an expressed sequence tag representing the cuticle-degrading serine protease gene (PII) from the nematophagous fungus *Artrhobotrys oviformis* by differential display technology. Genetics and Molecular Research, 7: 1200-1208.

NAHAR P, GHORMADE V, DESHPANDE MV. (2004). The extracellular constitutive production of chitin deacetylase in *Metarhizium anisopliae*: possible edge to entomopathogenic fungi in the biological control of insect pests. Journal of Invertebrte Pathology, 85: 80-88.

NAPOLITANO R, JUAREZ MP. (1997). Entomopathogenous fungi degrade epicuticular hydrocarbons of *Triatoma infestans*. Archives of Biochemistry and Biophysics, 344: 208-214.

NATIONAL ACADEMY OF SCIENCE. (1987). Report of the research briefing panel on biological control in managed ecosystems. National Academy Press, Washington, 206p.

O'FARRELL PH. (1975). High resolution two-dimensional electrophoresis of proteins. Journal of Biological Chemistry, 250: 4007-4021.

OH IS, PARK AR, BAE MS, KWON SJ, KIM YS, LEE JE, KANG NY, LEE S, CHEONG H, PARK OK. (2005). Secretome analysis reveals an *Arabidopsis* lipase involved in defense against *Alternaria brassicicola*. Plant Cell, 17: 2832-2847.

PAL S, ST LEGER RJ, WU LP. (2007). Fungal peptide destruxin A plays a specific role in suppressing the innate immune response in *Drosophila melanogaster*. Journal of Biological Chemistry, 282: 8969-8977.

PEDERSEN SK, SLOANE AJ, PRASAD SS, SEBASTIAN LT, LINDNER RA, HSU M, ROBINSON M, BYE PT, WEINGERGER RP, HARRY JL. (2005). An immunoproteomic approach for identification of clinical biomarkers for monitoring disease: application to cystic fibrosis. Molecular Cell Proteomics, 4: 1052-1060.

PEDRINI N, CRESPO R, JUAREZ MP. (2007). Biochemistry of insect epicuticle degradation by enomopathogenic fungi. Comparative Biochemistry and Physiology, part C, 146: 124-137.

PELL JK, EILENBERG J, HAJK AE, STEINKRAUS DC. (2001) Biology, ecology and pest management potential of entomophthorales. In: BUTT TM, JACKSON C, MAGAN N (Eds.) Fungal as biocontrol agents: progress, problems and potential. CAB International, Wallingford, p. 77-153.

PINTO AS, BARRETO CC, SCHRANK A, ULHOA CJ, VAINSTEIN MH. (1997). Purification and characterization of an extracellular chitinase from the entomopathogen *Metarhizium anisopliae*. Canadian Journal of Microbiology, 43: 322-327.

POLAR P, KAIRO MT, MOORE D, PEGRAM R, JOHN SA. (2005). Comparison of water, oils and emulsifiable adjuvant oils as formulating agents for *Metarhizium anisopliae* for use in control of *Boophilus microplus*. Mycopathologia, 160: 151-157.

QAZI SS, KHACHATOURIANS GG. (2007). Hydrated conidia of *Metarhizium anisopliae* release a family of metalloproteases. Journal of Invertebrate Pathology, 95: 48-59.

QUESADA-MORAGA E, SANTOS-QUIRÓS R, VALVERDE-GARCÍA P, SANTIAGO-ÁLVAREZ C. (2004). Virulence, horizontal transmission, and sub lethal reproductive effects of *Metarhizium anisopliae* (Anamorphic fungi) on the German cockroach (Blattodea: Blattellidae). Journal of Invertebrate Pathology, 87: 51-58.

RANGEL DEN, BRAGA GU, FLINT SD, ANDERSON AJ, ROBERTS DW. (2004). Variations in UV-B tolerance and germination speed of *Metarhizium anisopliae* conidia produced on insects and artificial substrates. Journal of Invertebrate Pathology, 87: 77-83.

ROBERT A, AL AIDROOS KM. (1985). Acid production by *Metarhizium anisopliae*: Effects on virulence against mosquitoes and on detection of *in vitro* amylase, protease, and lipase activity. Journal of Invertebrate Pathology, 45: 9-15.

ROBERTS DW, ST LEGER JR. (2004). *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. Advances in Applied Microbiology, 54: 1-70.

SASAKI SD, LIMA CA, LOVATO DV, JULIANO MA, TORQUATO RJS, TANAKA AS. (2008). BmSI-7, a novel subtilisin inhibitor from *Boophilus microplus*, with activity toward Pr1 proteases from the fungus *Metarhizium anisopliae*. Experimental Parasitology, 118: 214-220.

SCHOLTE EJ, NG´HABI K, KIHONDA J, TAKKEN W, PAAIJMANS K, ABDULLA S, KILLEEN GF, KNOLS BGJ. (2005). An entomopathogenic fungus for control of adult African malaria mosquitoes. Science, 308: 1641-1652.

SCHOLTE EJ, TAKKEN W, KNOLS BGJ. (2007). Infection of adult *Aedes aegypti* and *Ae. albopictus* mosquitoes with the entomopathogenic fungus *Metarhizium anisopliae*. Acta Tropica, 102: 151-158.

SCHRANK A, BASSANESI MC, PINTO Jr H, COSTA SV, BOGO M, SILVA MSN. (1993). Superoxide dismutases in the entomopathogenic fungus *Metarhizium anisopliae*. Ciência e Cultura, 45: 200-205.

SCREEN SE, ST LEGER RJ. (2000). Cloning, expression, and substrate specificity of a fungal chymotrypsin. Evidence for lateral gene transfer from an actinomycete bacterium. The Journal of Biological Chemistry, 275: 6689-6694.

SCREEN SE, HU G, ST LEGER RJ. (2001). Transformants of *Metarhizium anisopliae* sf. *anisopliae* overexpressing chitinase from *Metarhizium anisopliae* sf. *acridum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta*. Journal of Invertebrate Pathology, 78: 260-266.

SHAH P, ATWOOD JA, ORLANDO R, EL MUBAREK H, PODILA GK, DAVIS MR. (2009). Comparative proteomic analysis of *Botrytis cinerea* secretome. Journal of Proteome Research, in press.

SHAH PA, PELL JK. (2003). Entomopathogenic fungi as biological control. Applied Microbiology and Biotechnology, 61: 413-423.

SHAPIRO-ILAN DI, FUXA JR, LACEY LA, ONSTAD DW, KAYA HK. (2005). Definitions of pathogenicity and virulence in invertebrate pathology. Journal of Invertebrate Pathology, 88: 1-7.

SHIN GW, PALAKSHA KJ, KIM YR, NHO SW, CHO JH, HEO NE, HEO GJ, PARK SC, JUNG TS. (2007). Immunoproteomic analysis of capsulate and non-capsulate strains of *Lactococcus garvieae*. Veterinary Microbiology, 119: 205-212.

SILVA MV, SANTI L, STAATS CC, COSTA AM, COLODEL EM, DRIEMEIER D, VAINSTEIN MH, SCHRANK A. (2005). Cuticle-induced endo/exoacting chitinase CHIT30 from *Metarhizium anisopliae* is encoded by an ortholog of the chi3 gene. Research in Microbiology, 156: 382-392.

SILVA WOB. (2005). Lipases de *Metarhizium anisopliae*: purificação parcial, regulação e secreção durante o processo de infecção do carrapato bovino *Boophilus microplus*. Dissertação de Mestrado. Curso de Pós-graduação em Biologia Celular e Molecular (PPGBCM) da Universidade Federal do Rio Grande do Sul, Porto Alegre.

SILVA WOB, MITIDIERI S, SCHRANK A, VAINSTEIN MH. (2005). Production and extraction of an extracellular lipase from the entomopathogenic fungus *Metarhizium anisopliae*. Process Biochemistry, 40: 321-326.

SMITHSON SL, PATERSON IC, BAILEY AM, SCREEN SE, HUNT BA, COBB BD, COOPER RM, CHARNLEY AK, Clarkson JM. (1995). Cloning and characterization of a gene encoding a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. Gene, 166: 161-165.

STAATS CC. (2007). Estudo functional de genes do fungo entomopatogênico *Metarhizium anisopliae*. Tese de Doutroado. Curso de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul, Porto Alegre.

STANISÇUASKI F, FERREIRA-DA SILVA CT, MULINARI F, PIRES-ALVES M, CARLINI CR. (2005). Insectical effects of canatoxin on the cotton stainer bug *Dysdercus peruvianus* (Hemiptera: Pyrrhocoridae). Toxicon, 45: 753-760.

ST LEGER RJ, COOPER RM, CHARNLEY AK. (1986a). Cuticle-degrading enzymes of entomopathogenic fungi: Synthesis in culture on cuticle. Journal of Invertebrate Pathology, 48: 85-95.

ST LEGER RJ, CHARNLEY AK, COOPER RM. (1987). Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae*. Archives of Biochemistry and Biophysics, 253: 221-232.

ST LEGER RJ, BUTT TM, STAPLES RC, ROBERTS DW. (1990). Second messenger involvement in differentiation of the entomopathogenic fungus *Metarhizium anisopliae*. Journal of General Microbiology, 136: 1779-1789.

ST LEGER RJ, COOPER RM, CHARNLEY AK. (1991a). Characterization of chitinase and chitobiase produced by the entomopathogenic fungus *Metarhizium anisopliae*. Journal of Invertebrate Pathology, 58: 415-426.

ST LEGER RJ, ROBERTS DW, STAPLES RC. (1991b) A model to explain differentiation of appressoria by germlings of *Metarhizium anisopliae*. Journal of Invertebrate Pathology, 57: 299-310.

ST LEGER RJ, STAPLES RC, ROBERTS DW. (1992). Cloning and regulatory analysis of starvation-stress gene, ssgA, encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae*. Gene, 120: 119-124.

ST LEGER RJ, BIDOCHKA MJ, ROBERTS DW. (1994a). Isoforms of the cuticle-degrading Pr1 proteinase and production of a metalloproteinase by *Metarhizium anisopliae*. Archives of Biochemistry and Biophysics, 313: 1-7.

ST LEGER RJ, BIDOCHKA MJ, ROBERTS DW. (1994b). Characterization of a novel carboxypeptidase produced by the entomopathogenic fungus *Metarhizium anisopliae*. Archives of Biochemistry and Biophysics, 314: 392-398.

ST LEGER RJ, JOSHI L, BIDOCHKA RJ, RIZZO NW, ROBERTS DW. (1996a). Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Applied and Environmental Microbiology, 62: 1257-1264.

ST LEGER RJ, JOSHI L, BIDOCHKA MJ, ROBERTS DW. (1996c). Construction of an improved mycoinsecticide overexpressing a toxic protease. Proceedings of the National Academy of Sciences of the USA, 93: 6349-6354.

ST LEGER RJ, JOSHI L, ROBERTS DW. (1998). Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Applied and Environmental Microbiology, 64: 709-713.

ST LEGER RJ. (2008). Studies on adaptation of *Metarhizium anisopliae* to life in the soil. Journal of Invertebrate Pathology, 98: 271-276.

SUAREZ MB, SANZ L, CHAMORRO ML, REY M, GONZÁLEZ FJ, LLOBELL A, MONTE E. (2005). Proteomic analysis of secreted proteins from *Trichoerma harzianum*: identification of a fungal cell wall-induced aspartic protease. Fungal Genetics and Biology, 42: 924-934.

TIAGO PV, FUNGARO MH, FARIA MR, FURLANETO MC. (2004). Effects of double-stranded RNA in *Metarhizium anisopliae* var. *acridum* and *Paecilomyces fumosoroseus* on protease activities, conidia production, and virulence. Canadian Journal of Microbiology, 50: 335-339.

TOLEDO AV, DE REMES LENICOV AM, LOPEZ LASTRA CC. (2007). Pathogenicity of fungal isolates (Ascomycota: Hypocreales) against *Peregrinus maidis, Delphacodes kuscheli* (Hemiptera: Delphacidae), and *Dalbulus maidis* (Hemiptera: Cicadellidae), vectors of corn disease. Mycopathologia, 163: 225-232.

TSENG SC, LIU SY, YANG HH, LO CT, PENG KC. (2008). Proteomic study of biocontrol mechanisms of *Trichoderma harzianum* ETS 323 in response to *Rhizoctonia solani*. Journal of Agricultural and Food Chemistry, 56: 6914-6922.

TULLOCH M. (1976). The genus *Metarhizium*. Transactions of the British Mycological Society, 66: 407-411.

ULHOA CJ, PEBERDY JF. (1991). Regulation of chitinase synthesis in *Trichoderma harzianum*. Journal of General Microbiology, 137: 2163-2169.

WANG C, TYPAS MA, BUTT TM. (2002). Detection and characterization of *pr*1 virulent gene deficiencies in the insect pathogenic fungus *Metarhizium anisopliae*. FEMS Microbiology Letters, 213: 251-255.

WANG C, SKROBEK A, BUTT TM. (2003). Investigations on the destruxin production of the entomopathogenic fungus *Metarhizium anisopliae*. Journal of Invertebrate Pathology, 85: 168-174.

WANG C, HU G, ST LEGER RJ. (2005). Differential gene expression by *Metarhizium anisopliae* growing in root exudates and host (*Manduca sexta*) cuticle or hemolymph reveals mechanisms of physiological adaptation. Fungal Genetics and Biology, 42: 704-718.

WANG C, ST LEGER RJ. (2005). Developmental and transcriptional responses to host and nonhost cuticles by the specific locust pathogen *Metarhizium anisopliae* var. *acridum*. Eukaruotic Cell, 4: 937-947.

WANG Y, YANG L, XU H, LI Q, MA Z, CHU C. (2005). Differential proteomic analysis of proteins in wheat spikes induced by *Fusarium graminearum*. Proteomics, 5: 4496-4503.

WANG C, ST LEGER RJ. (2006). A collagenous protective coat enable *Metarhizium anisopliae* to evade insect immune responses. Proceedings of the National Academy of Sciences of the USA, 103: 6647-6652.

WANG C, ST LEGER RJ. (2007a). The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryotic Cell, 6: 808-816.

WANG C, ST LEGER RJ. (2007b). The *Metarhizium anisopliae* perilipin homolog MPL1 regulates lipid metabolism, appressorial turgor pressure, and virulence. The Journal of Biological Chemistry, 282: 21110-21115.

WANG C, ST LEGER RJ. (2007c). A scorpion neurotoxin increases the potency of a fungal insecticide. Nature Biotechnology, 25: 1455-1456.

WANG C, DUAN Z, ST LEGER RJ. (2008). MOS1 osmosensor of *Metarhizium anisopliae* is required for adaptation to infect host hemolymph. Eukaryotic Cell, 7: 302-309.

WILKINS MR, SANCHEZ JC, GOOLEY AA, APPEL RD, HUMPHERY-SMITH I, HOCHSTRASSER DF, WILLIAMS KL. (1995). Progress with proteome projects: why all proteins expressed by a genome should be identified and now to do it. Biotechnology & Genetic Engineering Reviews, 13: 19-50.

WU GS, SHORTT BJ, LAWRENCE EB, LEON J, FITZSIMMONS KC, LEVINE EB, RASKIN I, SHAH DM. (1997). Activation of host defense mechanisms by elevated production of H_2O_2 in transgenic plants. Plant Physiology, 115: 427-435.

WU Z, ZHANG W, LU C. (2008). Immunoproteomic assay of surface proteins of *Streptococcus suis* serotype 9. FEMS Immunology and Medical Microbiology, 53: 52-59.

XIA Y, CLARKSON JM, CHARNLEY AK. (2002). Trehalose-hydrolysis enzymes of *Metarhizium anisopliae* and their role in pathogenesis of the tobacco hornworm, *Manduca sexta*. Journal of Invertebrate Pathology, 80: 139-147.

ZHAO H, CHARNLEY AK, WANG Z, YIN Y, LI Z, LI Y, CAO Y, PENG G, XIA Y. (2006). Identification of an extracellular acid trehalase and its gene involved in fungal pathogenesis of *Metarhizium anisopliae*. Journal of Biochemistry, 140: 319-327.

ANEXOS

Lucélia Santi

Curriculum Vitae

Junho/2009

Dados Pessoais

Nome Lucélia Santi
Filiação João Santi e Edeni Rodrigues Santi
Nascimento 19/01/1978 - Bento Gonçalves/RS - Brasil

Formação Acadêmica/Titulação

2009 Pós-Doutorado.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2005 - 2009 Doutorado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Relação patógeno-hospedeiro: Análise bioquímica e proteômica da interação do fungo Metarhizium anisopliae e seus hospedeiros artrópodes, Ano de obtenção: 2009
Orientador: Marilene Henning Vainstein
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2004 - 2005 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Produção, caracterização e aplicação de extratos pectinolíticos produzidos por Penicillium oxalicum utilizando resíduos agroindustriais, Ano de obtenção: 2005
Orientador: Dra Marilene Henning Vainstein
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

1999 - 2003 Graduação em Licenciatura Em Ciências Biológicas.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

1999 - 2003 Graduação em Bacharelado em Ciências Biológicas- enf Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Otimização do processo de purificação de uma quitinase de Metarhizium anisopliae e produção de anticorpos policlonais
Orientador: Dra Marilene Henning Vainstein
Bolsista do(a): Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul

Formação complementar

1996 - 1996 Extensão universitária em Organização e montagem de um laboratório alternati.
Universidade de Caxias do Sul, UCS, Caxias Do Sul, Brasil

1996 - 1996 Extensão universitária em Biologia e comportamento das serpentes do Rio Gran.
Universidade de Caxias do Sul, UCS, Caxias Do Sul, Brasil

2000 - 2000 Produção e análise de produtos transgênicos.

Congresso Nacional de Genética, CNG, Brasil

2000 - 2000 Extensão universitária em Biologia molecular aplicada à clínica.
universidade federal de ciencias da saúde de porto alegre, UFCSPA, Brasil

2001 - 2001 Extensão universitária em Eletroforese bi-dimensional.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2002 - 2002 Extensão universitária em Seminário de Atualidades Genéticas.
Pontifícia Universidade Católica do Rio Grande do Sul, PUC RS, Porto Alegre, Brasil

2004 - 2004 Curso de curta duração em Seqüenciamento e análise de genomas II.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2007 - 2007 Curso de curta duração em Técnicas moleculares aplicadas à análise proteômic.
EMBRAPA - CENARGEN, CENARGEN, Brasil

2008 - 2008 Introduction to mass spectrometry and proteomics a.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

2009 - 2009 Curso de curta duração em Fundamentos de Análise Proteömica.
Fundação Oswaldo Cruz, FIOCRUZ, Rio De Janeiro, Brasil

Atuação profissional

1. Universidade Federal do Rio Grande do Sul - UFRGS

Vínculo institucional

2009 - Atual Vínculo: Bolsista recém-doutor , Enquadramento funcional: pós-doutorado, Regime: Dedicação Exclusiva

2005 - 2009 Vínculo: doutorado , Enquadramento funcional: aluno de pós-graduação, Regime: Dedicação Exclusiva

2004 - 2005 Vínculo: mestrado , Enquadramento funcional: aluno de pós-graduação, Regime: Dedicação Exclusiva

1999 - 2003 Vínculo: Outro , Enquadramento funcional: bolsista de iniciação , Carga horária: 20, Regime: Parcial

Atividades

05/2009 - Atual Pesquisa e Desenvolvimento, Laboratório de Biologia de Fungos de Importância Médica e Biotecnológica
Linhas de Pesquisa:
Análise proteômica de biofilmes produzidos pelo complexo Cryptococcus

11/2005 - 04/2009 Projetos de pesquisa, Laboratório de Biologia de Fungos de Importância Médica e Biotecnológica
Participação em projetos:
Estudo da interação patógeno-hospedeiro utilizando como modelo o fungo Metarhizium anisopliae e o inseto manchador do algodão Dysdercus peruvianus , Efeito de formulações contendo óleos do fungo Metarhizium anisopliae sobre o percevejo manchador do algodão Dysdercus peruvianus , Imunoproteômica de proteínas possivelmente envolvidas na interação entre o fungo Metarhizium

anisopliae e hospedeiros artrópodes , Detecção de proteínas da superfície do esporo de Metarhizium anisopliae

03/2004 - 10/2005 Pesquisa e Desenvolvimento, Laboratório de Biologia de Fungos de Importância Médica e Biotecnológica
Linhas de Pesquisa:
Produção e caracterização de pectinases produzidas por Penicillium oxalicum , Aplicação de pectinases produzidas por Penicillium oxalicum na extração de sucos de frutas

12/1999 - 12/2003 Pesquisa e Desenvolvimento, Laboratório de Biologia de Fungos de Importância Médica e Biotecnológica
Linhas de Pesquisa:
Construção de linhagens transgênicas de fungos filamentosos para o controle biológico , Purificação de quitinases de Metarhizium anisopliae , Controle biológico de Dysdercus peruvianus por Metarhizium anisopliae

2. Empresa Brasileira de Pesquisa Agropecuária - Embrapa

Vínculo institucional

1997 - 1999 Vínculo: Outro , Enquadramento funcional: bolsista de iniciação , Carga horária: 20, Regime: Parcial

Atividades

07/1997 - 06/1999 Estágio, Centro Nacional de Pesquisa de Uva e Vinho, Cnpuv Microbiologia
Estágio:
Seleção de leveduras e produtos de metabólitos

3. Universidade de Caxias do Sul - UCS

Vínculo institucional

1996 - 1997 Vínculo: Outro , Enquadramento funcional: bolsista de iniciação científica , Carga horária: 20, Regime: Parcial

Atividades

08/1996 - 06/1997 Pesquisa e Desenvolvimento, Instituto de Biotecnologia, Laboratório de Enobiotecnologia
Linhas de Pesquisa:
Os fatores killers de leveduras na indústria de vinificação

Linhas de pesquisa

1. Os fatores killers de leveduras na indústria de vinificação

2. Análise proteômica de biofilmes produzidos pelo complexo Cryptococcus

3. Aplicação de pectinases produzidas por Penicillium oxalicum na extração de sucos de frutas

4. Construção de linhagens transgênicas de fungos filamentosos para o controle biológico

5. Controle biológico de Dysdercus peruvianus por Metarhizium anisopliae

6. Produção e caracterização de pectinases produzidas por Penicillium oxalicum

7. Purificação de quitinases de Metarhizium anisopliae

Áreas de atuação

1. Microbiologia Aplicada
2. Bioquímica dos Microorganismos
3. Biologia Molecular
4. Enzimologia

Idiomas

Inglês Compreende Pouco , Fala Razoavelmente, Escreve Razoavelmente, Lê Bem

Espanhol Compreende Razoavelmente , Fala Pouco, Escreve Pouco, Lê Razoavelmente

Prêmios e títulos

2004 3o. lugar na IV Maratona de Empreendedorismo, UFRGS

2003 Destaque - XV Salão de Iniciação Científica, UFRGS

Produção em C, T& A

Produção bibliográfica
Artigos completos publicados em periódicos

1. AB Becker-Ritt, MARTINELLI, A.H.S., MITIDIERI, S., FEDER, V., WASSERMANN, G.E., SANTI, L., VAINSTEIN, Marilene Henning, OLIVEIRA, J.T.A., FIUZA, L., PASQUALI, G., CARLINI, C.R.
Antifungal activity of plant and bacterial ureases. Toxicon. , v.50, p.971 - 983, 2007.

2. SILVA, Márcia Vanusa da, SANTI, L., STAATS, C. C., COSTA, A. M., COLODEL, E. M., DRIEMEIER, D., VAINSTEIN, Marilene Henning, SCHRANK, Augusto
The cuticle-induced endo/exo acting-chitinase CHIT30 from Metarhizium anisopliae is encoded by an ortholog of the chi3 gene. Research in Microbiology (Paris). , v.156, p.382 - 392, 2005.

3. BARATTO, César Miltom, SILVA, Márcia Vanusa da, SANTI, L., PASSAGLIA, Luciane, SCHRANK, Irene Silveria, VAINSTEIN, Marilene Henning, SCHRANK, Augusto
Expression and characterization of the 42kDa chitinase of the biocontrol fungus Metarhizium anisopliae in Escherichia coli. Canadian Journal of Microbiology. , v.49, p.723 - 726, 2003.

4. PECCI, M. L. P. G., BOGO, M. R., SANTI, L., MORAES, C. K., CORRÊA, C. T., VAINSTEIN, Marilene Henning, SCHRANK, Augusto
Characterization of mycoviruses and analyses of chitinases secretion in the biocontrol fungus

Metarhizium anisopliae. Current Microbiology. , v.45, p.334 - 339, 2002.

Artigos aceitos para publicação

1. SILVA, W. O. B., SANTI, L., BERGER, M., Pinto, A. F. M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Characterization of a spore surface lipase from the biocontrol agent Metarhizium anisopliae. Process Biochemistry. , 2009.

Trabalhos publicados em anais de eventos (resumo)

1. SILVA, W. O. B., SANTI, L., BERGER, M., Pinto, A. F. M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Characterization of spore surface lipase from the biocontrol agent Metarhizium anisopliae In: X Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular, 2008, Porto Alegre.
X Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. , 2008. p.27 -

2. SANTI, L., SILVA, W. O. B., BERGER, M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Detecção de diferentes atividades enzimáticas de proteínas da superfície do esporo do fungo entomopatogênico e acaricida Metarhizium anisopliae In: VIII ENZITEC – Seminário Brasileiro de Tecnologia Enzimática, 2008, Rio de JAneiro.
VIII ENZITEC – Seminário Brasileiro de Tecnologia Enzimática. , 2008. p.151 -

3. SANTI, L., SILVA, W. O. B., BERGER, M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Enzymatic profile of spore surface proteins from Metarhizium anisopliae In: . X Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular, 2008, Porto Alegre.
. X Reunião Anual do Programa de Pós-Graduação em Biologia Celular e Molecular. , 2008. p.24 -

4. SILVA, W. O. B., BERGER, M., SANTI, L., Pinto, A. F. M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Purificação e caracterização de uma lipase de superfície de esporo de Metarhizium anisopliae In: VIII ENZITEC – Seminário Brasileiro de Tecnologia Enzimática, 2008, Rio de Janeiro.
VIII ENZITEC – Seminário Brasileiro de Tecnologia Enzimática. , 2008. p.152 -

5. SANTI, L., SILVA, W. O. B., BERGER, M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Enzymatic profile of spore surface proteins from Metarhizium anisopliae In: XXXVI Reunão anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2007, Salvador.
Livro de resumos da XXXVI Reunião anual da SBBq. , 2007.

6. SILVA, W. O. B., BERGER, M., SANTI, L., Pinto, A. F. M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Purification of spore surface lipase from Metarhizium anisopliae In: XXXVI Reunão anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2007, Salvador.
Livro de resumos da XXXVI Reunião anual da SBBq. , 2007.

7. SANTI, L., SILVA, W. O. B., VAINSTEIN, Marilene Henning
Aplicação de preparados pectinolíticos de Penicillium oxalicum na extração de sucos de frutas In: VII Seminário Brasileiro de Tecnologia Enzimática, 2006, Caxias do Sul.

Livro de resumos do VII Seminário Brasileiro de Tecnologia Enzimática. , 2006. p.103 -

8. SILVA, W. O. B., SANTI, L., BERGER, M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Detecção de diferentes atividades enzimáticas de proteínas da superfície do esporo do fungo entomopatogênico e acaricida Metarhizium anisopliae In: VII seminário Brasileiro de Tecnologia Enzimática, 2006, Caxias do Sul.
Livro de Resumos do VII seminário Brasileiro de Tecnologia Enzimática. , 2006. p.60 -

9. SILVA, W. O. B., SANTI, L., BERGER, M., Pinto, A. F. M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Partial purification of a spore surface lipase from the entomopathogenic and acaricide fungus Metarhizium anisopliae In: XXXV Reunão anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2006, Águas de Lindóia.
Livro de resumos da XXXV Reunião anual da SBBq. , 2006.

10. SANTI, L., SILVA, W. O. B., BERGER, M., GUIMARAES, J. A., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Partial purification of spore surface chitinases from the entomopathogenic fungus Metarhizium anisopliae In: XXXV Reunão anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2006, Águas de Lindóia.
Livro de resumos da XXXV Reunião anual da SBBq. , 2006.

11. SILVA, W. O. B., SANTI, L., CORREA, A. P. F., SILVA, L. A. D., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Atividade lipolítica de Metarhizium anisopliae durante o processo de infecção do carrapato bovino Boophilus microplus In: VIII Symposium on Enzymatic Hydrolysis of Biomass, 2005, Maringá.
Livro de resumos do VIII Symposium on Enzymatic Hydrolysis of Biomass. , 2005. p.145 -

12. SANTI, L., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Estudo da interação patógeno-hospedeiro utilizando como modelo o fungo entomopatogênico Metarhizium anisopliae e o inseto-praga Dysdercus peruvianus In: VII Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular, 2005, Porto Alegre.
VII Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular. , 2005. p.90 -

13. SANTI, L., SILVA, W. O. B., VAINSTEIN, Marilene Henning
Produção de pectinases por Penicillium oxalicum utilizando resíduos agroindustriais In: VIII Symposium on Enzymatic Hydrolysis of Biomass, 2005, Maringá.
Livro de resumos do VIII Symposium on Enzymatic Hydrolysis of Biomass. , 2005. p.169 -

14. SILVA, Márcia Vanusa da, SANTI, L., COSTA, A. C. O., VAINSTEIN, Marilene Henning, SCHRANK, Augusto
Clonagem e caracterização do gene que codifica a quitinase CHIT30 de Metarhizium anisopliae In: XXIII Reunião de Genética de Microrganismos, 2002, Pirenópolis.
Anais da XXIII Reunião de Genética de Microrganismos. , 2002. p.81 - 81

15. SANTI, L., SILVA, Márcia Vanusa da, SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Purificação parcial de quitinases extracelulares produzidas por Metarhizium anisopliae In: XIV Salão de Iniciação Científica, 2002, Porto Alegre.
. , 2002.

16. SANTI, L., SILVA, Márcia Vanusa da, SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Purificação parcial de uma quitinase extracelular produzida por Metarhizium anisopliae In: XIII Salão de Iniciação Científica, 2002, Porto Alegre.

. , 2002.

17. SANTI, L., SILVA, Márcia Vanusa da, SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Regulação da secreção de quitinases do entomopatógeno e acaricida Metarizium anisopliae In: XXIII Reunião de Genética de Microrganismos, 2002, Pirenópolis.
Anais da XXIII Reunião de Genética de Microrganismos. , 2002. p.79 - 79

18. SANTI, L., SILVA, Márcia Vanusa da, SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Purification of an extracellular chitinase from the entomopathogen Metarhizium anisopliae In: XXX Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2001, Caxambú- MG.
Programa e Resumos da XXX Reunião Anual -SBBq. São Paulo: SBBq, 2001. p.66 - 66

19. SANTI, L., MORAES, C. K., SCHRANK, Augusto, VAINSTEIN, Marilene Henning
Análise da secreção de quitinases e proteases em diferentes linhagens do fungo entomopatogênico Metarhizium anisopliae. Existe diferença na infectividade entre as linhagens infectadas ou não infectadas por micovírus? In: 46. Congresso Nacional de Genética, 2000, Águas de Lindóia.
Livro de resumos. , 2000. v.23. p.341 - 341

20. SANTI, L., MORAES, C. K., VAINSTEIN, Marilene Henning, SCHRANK, Augusto
Purificação de quitinases do fungo entomopatogênico Metarhizium anisopliae In: XII Salão de Iniciação Científica, 2000, Porto Alegre.
. Porto Alegre: UFRGS, 2000.

21. TORESAN, S. O. P., SANTI, L., MARIS, A. S. P., BONATO, D., HENRIQUES, J. A. P.
O produto do gene RAD16 de Saccharomyces cerevisiae é indispensável para a indução de RNR, genes induzíveis por danos ao DNA. In: 43. Congresso Nacional de Genética, 1997, Goiânia.
Revista Brasileira de Genética. , 1997. v.20. p.245 - 245

Artigos em revistas (Magazine)

1. FRANCESCHINI, M., GUIMARÃES, A. P., CAMASSOLA, M., FRAZZON, A. P., BARATTO, C. M., SILVA, Márcia Vanusa da, DUTRA, V., SANTI, L., VAINSTEIN, Marilene Henning, SCHRANK, Augusto
Biotecnologia aplicada ao controle biológico. Biotecnologia Ciência e Desenvolvimento. Brasil, p.33 - 39, 2001.

Orientações e Supervisões

Orientações e Supervisões em andamento

Iniciação científica

1. Gabriel Perotoni. **Isolamento e seleção de bactérias ambientais para o controle biológico associado ao fungo Metarhizium anisopliae do inseto manchador do aglodão Dysdercus peruvianus**. 2008. Iniciação científica (Ciências Biológicas) - Universidade Federal do Rio Grande do Sul

Eventos

Participação em eventos

1. Apresentação de Poster / Painel no(a) **VIII Seminário Brasileiro de Tecnologia Enzimática**

(ENZITEC), 2008. (Seminário)
Detecção de diferentes atividades enzimáticas de proteínas da superfície do esporo do fungo entomopatogênico e acaricida Metarhizium anisopliae.

2. Apresentação de Poster / Painel no(a) **XXXVI Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology and 10th International Union of Biochemistry and Molecular Biology**, 2007. (Congresso)
Enzymatic profile of spore surface proteins from Metarhizium anisopliae.

3. Apresentação de Poster / Painel no(a) **XXXV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular**, 2006. (Congresso)
Partial purification of spore surface chitinase from the entomopathogenic fungus Metarhizium anisopliae.

4. Apresentação de Poster / Painel no(a) **VII Seminário Brasileiro de Tecnologia Enzimática - ENZITEC**, 2006. (Seminário)
VII Seminário Brasileiro de Tecnologia Enzimática - ENZITEC.

5. Apresentação de Poster / Painel no(a) **VIII Seminário de Hidrolise Ezimática de Biomassas (SHEB)**, 2005. (Seminário)
Produção de pectinases por Penicillium oxalicum utilizando resíduos agroindustriais.

6. Apresentação de Poster / Painel no(a) **VI Seminário Brasileiro de Tecnologia Enzimática (ENZITEC)**, 2004. (Seminário)
Isolamento, seleção e produção de poligalacturonase por fungos filamentosos em meio líquido e semi-sólido.

7. Apresentação de Poster / Painel no(a) **XXIV Reunião de Genética de Microrganismos**, 2004. (Congresso)
Produção de poligalacturonase por fungos filamentosos em meio líquido e semi-sólido.

8. Apresentação de Poster / Painel no(a) **XXIII Reunião de Genética de Microrganismos**, 2002. (Congresso)
Clonagem e caracterização do gene que codifica a quitinase CHIT30 de Metarhizium anisopliae.

9. **XI Reunião Estadual de Biotecnologia Vegetal**, 2002. (Encontro)

10. Apresentação de Poster / Painel no(a) **XXX Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular**, 2001. (Congresso)
Purification of an extracellular chitinase from the entomopathogen Metarhizium anisopliae.

11. Apresentação de Poster / Painel no(a) **46º Congresso Nacional de Genética**, 2000. (Congresso)
Análise da secreção de quitinases e proteases em diferentes linhagens do fungo entomopatogênico Metarhizium anisopliae, Existe diferença na infectividade entre as linhagens infectadas ou não infectadas por micovírus?.

12. **XXIII Congresso Brasileiro de Zoologia**, 2000. (Congresso)
Organização de evento

1. SANTI, L.
VII Seminário Brasileiro de Tecnologia Enzimática, 2006. (Congresso, Organização de evento)