ALINE RAFAELA MOURA GARCIA

# ENTOMOPATHOGENIC FUNGI FOR BIOLOGICAL CONTROL OF CHAGAS DISEASE VECTOR *Rhodnius prolixus*

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Magister Scientiae*.

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APROVADA: 19 de Julho de 2013.

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Raul Narciso Carvalho Guedes Davi Mesquita de Macedo

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Gustavo Ferreira Martins

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Simon Luke Elliot
(Orientador)

“I learned that courage was not the absence of fear, but the triumph over it. The brave man is not he who does not feel afraid, but he who conquers that fear”.
Nelson Mandela

“Ensinar não é uma função vital, porque não tem o fim em si mesma; a função vital é aprender”. Aristóteles

À minha família

Dedico

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# ÍNDICE

## RESUMO

GARCIA, Aline Rafaela Moura. M.Sc., Universidade Federal de Viçosa, julho de 2013. **Fungos entomopatogênicos para o controle biológico do vetor da doença de Chagas** *Rhodnius prolixus*. Orientador: Simon Luke Elliot.

Parasitos transmitidos por insetos vetores são grandes problemas na saúde pública. Os métodos convencionais de controle, inseticidas químicos, não são sustentáveis por longos períodos, geram forte pressão de seleção às populações de insetos, ocasionando resistência, além de possíveis danos ambientais e à saúde humana. Fungos entomopatogênicos apresentam potencial para o controle de doenças transmitidas por insetos vetores. Nesse sentido, o objetivo deste trabalho foi avaliar a patogenicidade de isolados de *Metarhizium anisopliae* e *Beauveria bassiana* a *Rhodnius prolixus* (Reduviidae) e investigar a possibilidade de uso desses patógenos no manejo da doença de Chagas. Para isso, realizamos três ensaios em laboratório para: i) avaliar a patogenicidade e escolher isolados infectivos a triatomíneos, ii) investigar a virulência de isolados através de ensaio de concentração e iii) avaliar o efeito da temperatura a insetos infectados com fungo. Através desses ensaios, observamos que isolados de *M.anisopliae* mostraram-se mais virulentos às ninfas de *R. prolixus* do que os isolados de *B. bassiana*, inclusive em baixa concentração de esporos. Também observamos que o tempo de vida de ninfas infectadas com os isolados URPE-11 e ENT-1, *M. anisopliae* e *B. bassiana*, respectivamente, foi reduzido com o aumento da temperatura. No entanto, os resultados nos indicam que ENT-1 pode ser mais efetivo que URPE-11 tanto em altas, quanto em baixas temperaturas. No campo, isolados capazes de infectar o hospedeiro em condições adversas de temperatura são preferíveis, no entanto, outros aspectos desses isolados ainda precisam ser avaliados para confirmar a possibilidade de utilização desses isolados para o controle e manejo de vetores da doença de Chagas.

## ABSTRACT

GARCIA, Aline Rafaela Moura. M.Sc., Universidade Federal de Viçosa, July, 2013. **Entomopathogenic fungi for biological control of Chagas disease vector** *Rhodnius prolixus***.** Advisor: Simon Luke Elliot.

Parasites transmitted by insects are major problems in public health. Conventional methods of control, chemical insecticides, are not sustainable for long periods, generate strong selection pressure on insect populations, for resistance, and can cause damage to the environment and human health. Entomopathogenic fungi have potential for control and management of diseases by insect vectors. In this sense, the objective of this study was to evaluate the pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* to *Rhodnius prolixus* (Reduviidae) and the possibility of using these pathogens for the management of Chagas disease. For this, we conducted three preliminary tests in laboratory to: i) assess the pathogenicity and choose infective isolate against triatomines, ii) investigate the virulence of isolates by assaying concentration on mortality and iii) to evaluate the effect of temperature on insects infected with fungus. Through these trials, we observed that isolates of *M. anisopliae* were more virulent to nymphs of *R. prolixus* than isolates of *B. bassiana*, even at low spore concentration. We also observed that the longevity of nymphs infected with the isolate URPE-11 and ENT-1, *M. anisopliae* and *B. bassiana*, respectively, was reduced with increasing temperature. However, ENT-1 showed to be more effective than URPE-11 both at high and low temperatures. In the field, isolates able to infect the host in adverse temperature conditions are preferable, however, other aspects of these isolates must be evaluated to confirm the possibility of using them for control and management of vectors of Chagas disease.

# INTRODUÇÃO GERAL

Insetos são vetores de inúmeros parasitos de doenças em seres humanos como dengue, malária, doença do sono, leishmaniose e doença de Chagas. De modo geral, o controle das doenças transmitidas por insetos é feito através de inseticidas, um importante componente em programas de controle vetorial, sobretudo por fornecerem um meio rápido e eficaz de redução das populações de insetos (Novak & Lampman 2001). No entanto, devido a preocupações sobre o amplo espectro de ação, supressão de inimigos naturais, efeitos nocivos à saúde humana, poluição do meio ambiente e forte pressão seletiva sobre os insetos, o uso de inseticidas tem sido amplamente discutido e questionado (Marcondes 2011). Por estas razões, táticas biológicas para o controle de insetos tem sido potencialmente demostradas através do uso de bactérias endossimbiontes, manipulações genéticas, predadores e patógenos (Chapman 1974; Scholte *et al*. 2004b; Moreira *et al*. 2009; Iturbe-Ormaetxe *et al*. 2011).

Nos últimos anos, fungos entomopatogênicos estão sendo estudados como ferramenta potencial para o controle de doenças como malária e dengue (Scholte *et al*. 2003; Blanford *et al*. 2005; Scholte *et al*. 2007). Esses patógenos podem infectar insetos em todos os seus estágios de desenvolvimento, pois a infecção ocorre através do contato dos esporos com a cutícula (Thomas & Read 2007). Esses microorganismos podem desempenhar papel importante, auxiliando na redução da capacidade vetorial e até mesmo inibindo o desenvolvimento de agentes etiológicos transmitidos por insetos vetores (Scholte *et al*. 2003; Scholte *et al*. 2004a, Blanford *et al*. 2005; Scholte *et al*. 2007; Achonduh & Tondje 2008), além de serem infectivos a populações resistentes a inseticidas. No campo, a eficácia e persistência desses patógenos, podem ser afetadas principalmente por fatores ambientais, como umidade e temperatura. Por esse motivo, é

essencial a seleção de isolados que sejam eficazes e persistentes a condições ambientais adversas.

A doença de Chagas representa um importante problema de saúde pública no continente americano (Coura & Pinto Dias 2009). A transmissão vetorial é um dos importantes mecanismos pelo qual os seres humanos são infectados pelo *Trypanosoma cruzi*, protozoário dessa doença (Dias *et al*. 2002). A transmissão do parasito ocorre através das fezes contaminadas de triatomíneos (Hemiptera: Reduviidae) popularmente conhecidos como barbeiros (Marcondes 2011). Várias espécies são vetores nas Américas e no Brasil, sendo as dos gêneros *Rhodnius* (*R. prolixus*), *Triatoma* (*T. infestans*) e *Panstrongylus* (*P. megistus*) os mais importantes. De acordo com a Organização Mundial de Saúde cerca de 12 milhões de pessoas estão infectadas e mais de 28 milhões em risco em países endêmicos (Guhl & Lazdins-Helds 2007).

A doença de Chagas tinha sido limitada às populações que vivem em áreas pobres e em zonas rurais; porém, com a migração de pessoas infectadas para as zonas urbanas, a doença começou a se espalhar em diferentes regiões não endêmicas, através de alimentos contaminados, transplante de órgãos, transfusão de sangue, transmissão congênita, aleitamento materno ou acidentalmente (Dias *et al*. 2011). A principal forma de controle vetorial é feito através do uso de insecticidas piretróides sintéticos nas casas infestadas e seu entorno (Dias & Schofield 1999). O uso de inseticida tem reduzido populações de triatomíneos em algumas regiões, mas isso não tem impedido a mobilidade dos vetores e como consequência, a reinfestação de domícilios tratados com inseticidas e o surgimento de populações resistentes a estes compostos (Zerba, 1999; Vassena *et al*. 2000; Ramsey *et al*. 2003).

Nesse contexto, ferramentas alternativas precisam ser investigadas. Considerando trabalhos já realizados com outros insetos vetores e com triatomíneos, os fungos entomopatogênicos são fortes candidatos ao controle de doença e manejo da

resistência a inseticidas. Por este motivo o objetivo desde estudo foi investigar o potencial uso de fungos entomopatogênicos para o manejo da doença de Chagas.

No capítulo 1 da dissertação, fazemos uma breve revisão dos aspectos que envolvem doenças transmitidas por insetos vetores, das principais experiências que envolvem o uso de fungos entomopatogênicos e perspectivas para a utilização no controle da doença de Chagas. No capítulo 2, nosso objetivo foi testar a patogenicidade e virulência de dez isolados de fungos entomopatogênicos para o controle do vetor da doença de Chagas, *Rhodnius prolixus*. Para isso, três ensaios foram realizados para avaliar a patogenicidade e virulência de isolados de *Beauveria bassiana* e *Metarhizium anisopliae*, efeito na sobrevivência a diferentes doses e efeito da temperatura na infecção por estes patógenos a *R. prolixus*.

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# CAPÍTULO 1

**Literature Review**

## Introduction

Pathogens transmitted by insect vectors cause morbidity and mortality of millions of people around the world. Diseases such as malaria, leishmaniasis, trypanosomiasis, Chagas disease, and others represent an enormous burden on public health, especially in tropical and subtropical regions (Hotez *et al*. 2006, 2007). The epidemiologies of these diseases are mainly associated with factors such as the condition of populations that live in endemic areas, the biology and behavior of the vector insects and also environmental factors (Marcondes 2011). Ecological and climatic changes resulting from land use, human travel, urbanization and suppression of forest areas, also create opportunities for increased vector populations and consequently the cycle of transmission of pathogens and parasites that cause disease (Adams & Kapan 2009; Paaijmans *et al*. 2009; Paaijmans *et al*. 2010; Colwell *et al*. 2011; Paaijmans & Thomas 2011).

The control of vectors in public health programs is based on the elimination of vector populations, supported by use of insecticides. However, there are concerns of insecticide impacts on the environment and human health. Furthermore, this method of control may not be sustainable in the long term because of the selective pressure on insect populations, leading to insecticide resistance (WHO 2010; Hunt *et al*. 2011). These concerns have led to an increased interest in biological methods for the control of vector-borne diseases (Thomas & Read 2007).

Biological methods consist of the utilization of natural enemies of targeted insects to achieve effective vector management (Vega & Kaya 2012). Pathogens that cause disease in insects provide an alternative method to manage populations of insects and diseases by reducing the vectorial capacity and even inhibit the development of etiological agents transmitted by insects. These include four main groups: virus,

bacteria, protozoa and fungi (Alves 1998; Scholte *et al*. 2003; Scholte *et al*. 2004; Blanford *et al*. 2005; Scholte *et al*. 2007; Achonduh & Tondje 2008).

Effective activity of bacteria *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) opened the possibility of using these microorganisms for controlling vector larvae (Lacey 2007; Paris *et al*. 2011). *Bacillus sphaericus* has been used for controlling larvae of *Culex pipiens*, *C. quinquefasciatus* and *Anopheles* spp. larvae while *B. thuringiensis israelensis* has a broader application including against larvae of *Aedes* and *Simulium* (Becker *et al*. 2010). However, two problems are associated with use of *Bacillus*: low residual activity and the arise of resistance (Becker & Ludwig 1993; Tabashnik 1994).

More recently *Wolbachia*, a bacteria endosymbiont, has been used at the field level as an alternative for dengue control (Walker *et al*. 2011). A strain of this bacterium originating from *Drosophila melanogaster* (*w*MelPop) was inserted in to *Aedes aegypti,* which does not naturally host *Wolbachia* (McMeniman *et al*. 2008). Mosquitoes containing the bacteria partially block the dengue virus, Chikungunya virus (a pathogen transmitted by *A.* aegypti mosquitoes that causes acute and chronic articular manifestations) and also the avian malaria parasite, *Plasmodium gallinaceum* (Moreira *et al*. 2009; Bian *et al*. 2010). Current studies show that *P. berghei* (Kambris *et al*. 2010) and *P. falciparum* are blocked in the presence of these bacteria in *Anopheles gambiae* (Hughes *et al*. 2011). This may open important possibility to control malaria and viral diseases (Iturbe-Ormaetxe *et al*. 2011).

**Entomopathogenic fungi and vector-borne diseases**

The most widespread insect pathogenic fungal are found in the order Hypocreales of the Phylum Ascomycota (Charnley & Collins 2007). In recent years

there has been an increase in interest on these pathogens for biological control of vector-borne diseases particularly with *Metarhizium* sp. and *Beauveria* sp. strains ( Scholte *et al*. 2003; Blanford *et al*. 2005).

These microorganisms are natural enemies of many insect species and act as important regulators in natural populations (Goettel *et al*. 2010). Effects on survival and capacity of the vectors to transmit parasites are the main reasons that have led to explore their potential for controlling insects of medical importance instead of relaying on (or in conjunction with) conventional of insecticide use (Scholte *et al*. 2007; Thomas & Read 2007; Achonduh & Tondje 2008).

According to Alves (1998), the great genetic variability of these entomopathogens can be considered one of the main advantages of microbial control. With the use of appropriate techniques it is possible to select or engineer highly virulent isolates or isolates that can express molecules to block agents, such as viruses and protozoa, transmitted by vectors (Wang & St Leger 2007). However, other authors support the idea that fungus does not necessarily need to be highly virulent, but capable of inducing disease in the insect and preventing parasite transmission (Thomas & Read 2007). Nevertheless, the identification of suitable fungal pathogen for the development of a mycoinsecticide can be complex because of the effectiveness that depends on intrinsic characteristics including virulence, insect host and environmental factors as tolerance to desiccation, temperature and ultraviolet radiation (Inglis *et al*. 2001).

Therefore, many studies have been performed to optimize the formulation and release methods of entomopathogenic fungi to attenuate problems in the field (Lwetoijera *et al*. 2010; Mnyone *et al*. 2010; Farenhorst *et al*. 2011). Efficacy and persistence through time are other heavily investigated issues (Farenhorst *et al*. 2010; Howard *et al*. 2010a; Kikankie *et al*. 2010; Paula *et al*. 2011).

The advantages of using fungi in biocontrol can include the possibility a degree of specificity to the host, low effect for beneficial insect predators, limited hazards to the environment or the health of mammals which is normally affected by insecticide applications (Goettel *et al*. 2010). On the other hand, some disadvantages are: very slow killing rate, necessity for specific conditions for use, high production costs, short-shelf life of spores and also potential risks to immunosuppressed people (Kanzok & Jacobs-Lorena 2006).

**Action of fungi in their hosts**

The insect cuticle is the first barrier of defense against infection, but a combination of mechanical pressure and the action of degradative enzymes generally proteinases, lipases and peptidases allows the fungus to infect the host (Jarrold *et al*. 2007). The first step is the adherence of spores on the cuticle, then the conidia germinate and the germ tube and appressorium with penetration peg are produced. Once the penetrative peg tube has passed through the cuticle and epidermis, the fungus can proliferate in the hemocoel. Then, they may produce toxins and grow either as blastospores (similar to yeast cells) or hyphal bodies (Thomas & Read 2007). Under appropriate conditions, particularly high relative humidity, the fungus grows in the host and external conidia are produced upon host death ( Hajek & St. Leger 1994; Goettel *et al*. 2010).

**Biological control and management of mosquitoes**

Mosquitoes are important vectors of parasites in humans including virus, protozoans and helminths. Among these are: malaria, transmitted by *Anopheles*

mosquitoes; leishmaniasis by sandflies; yellow fever and dengue, both mainly transmitted by the mosquito *Aedes aegypti*. Malaria is responsible for about 800.000 deaths each year with more than 200 million cases per year (WHO 2010) and dengue about 50 million cases annually (WHO 2009) in tropical and sub-tropical regions. Extensive use of insecticides against mosquitoes have caused strong pressure on insect populations resulting in the development of insecticide resistance (Nauen 2007). In recent years, alternative methods have been investigated to minimize the dependency of conventional methods of control, including studies that explore the potential use of entomopathogenic fungi.

Studies emphasize the selection of fungi isolates especially for the control of adult and immature stages of *Culex* sp., *Aedes* sp. and *Anopheles* sp., which can affect negatively their fecundity, longevity and feeding capacity (Scholte *et al*. 2006; Blanford *et al*. 2012). According to Scholte *et al*. (2003), the lifespans of *An. gambiae* and *C. quinquefasciatus* were reduced with an increase in conidia concentration of *M. anisopliae*, independent of exposure time (24 or 48 hrs). Moreover, given the high resistance the eggs of *Anopheles* sp. and *A. aegypti* in the dry season, the use of *M. anisopliae* and *B. bassiana* as ovicides (Albernaz *et al*. 2009; Luz *et al*. 2011; Leles *et al*. 2012) has also been proposed.

Another approach is the investigation of sublethal effects caused by these microorganisms to their hosts that might prevent the parasites transmission. Blanford *et al*. (2005) showed that reduction on survival of *An. stephensi* infected with *B. bassiana* can stop the *P. chabaudi* development, thus making it impossible to be transmitted by vector. Reduced blood feeding and egg laying reduction in *An. gambiae* also suggest that the infection by *M. anisopliae* may have an impact on *Plasmodium* transmission, perhaps due to an inability of infected insects to fly (Scholte *et al*. 2006).

In the current scenario, management could be changed from single to integrated control, combining microbial pesticides and conventional methods. Some isolates are equally pathogenic and virulent to insecticide-resistant and susceptible strains of *An. arabiensis* (Kikankie *et al*. 2010). Farenhorst *et al*. (2010) suggest the co-application of insecticides and fungi as an alternative to mitigate the problem of insecticide resistance as fungal infection caused 100% mortality rates in *An. gambiae* exposed to permethrin nine days after inoculation. Formulations of propagules of entomopathogenic fungi for field use have been guided by the need to improve the product shelf-life, efficacy and their physical characteristics for application.

**Other vectors**

Other vectors are susceptible to entomopathogenic fungi such as *Phlebotomus papatasi* (Diptera: Psychodidae). In laboratory conditions when exposed to *B. bassiana* spores on dry filter papers, this insects exhibited 100% of mortality after 4-6 days of exposure and *Lutzomyia longipalpis* by 4 days (Warburg 1991). Significant rates of larvae mortality, decreased adult longevity and reduced oviposition were found in *L. longipalpis* infected with *M. anisopliae* (Amora *et al*. 2010). Tse-tse flies (Diptera: Glossinidae), vectors of the sleeping sickness (human African trypanosomiasis), are also susceptible to *B. bassiana* and *M. anisopliae*. These fungi have reported to be virulent to larvae and adults of *Glossina morsitans* in the laboratory (Kaaya *et al*. 1991; Kaaya & Munyinyi 1995; Maniania 2002). More recently a preliminary evaluation of *B. bassiana* for bed bug control was carried out by Barbarin *et al*. (2012). All of these studies are basically about pathogenicity with the aim to select infective strains for insects.

**Entomopathogenic fungi for managing Chagas disease**

Triatomines (Hemiptera: Reduviidae) are vectors of *T. cruzi*, the causal agent of Chagas' disease. Insects acquire the parasite through infected blood during feeding. Control programs target the interruption of vectorial transmission by means of house spraying with insecticides (Coura & Pinto Dias 2009). However, the current methods of control are not sustainable for long periods. Insects resistant to the pyrethroid, deltamethrin, have been detected in some countries of South America associated with one of the most important vectors of Chagas disease *T. infestans* (Orihuela *et al*. 2008).

A number of isolates of entomopathogenic fungi have been shown to be infective to triatomines and can potentially be an alternative tool for controlling of Chagas disease. Pathogenicity of *M. anisopliae*, *M. flavoviride* var. *pemphigi*, *M. robertsii*, *I. cateniannulata* and *B. bassiana* have been evaluated against *Rhodniu*s *prolixu*s and *T. infestans* in the laboratory (Romaña & Fargues 1987; Rocha & Luz, 2011) and some attempts have been made to use oil-formulated *B. bassiana* under field and semi-field conditions in Central Brazil against *T. sordida* (Luz *et al*. 2004) with a decrease on insect survival next to 25 days after application in peridomestic areas on farms.

There are many factors that can affect the efficacy of biopesticides, such as slow speed of kill, moderate efficacy, poor storage characteristics, lack of field persistence after application, and expense (Goettel *et al*. 2010). Many of these factors could be overcome by strain selection, genetic improvement (Screen *et al*. 2001), formulation (Wraight *et al*. 2001) and application (Bateman & Chapple 2001). In addition, environmental factors such as solar radiation, temperature, water availability, precipitation and wind can be barriers to the use of entomopathogenic fungi (Inglis *et al*.

2001). However, compatibility between formulation and application techniques may assist to break these barriers.

Thus, considering the work so far performed for the control of many insect vectors, especially with mosquitoes, new prospects for the control of Chagas disease can arise through the use of entomopathogenic fungi. Furthermore, more studies are necessary to find pathogens with characteristics that permit the use of these tools in the field. With information about the behavior of infected insects it will possible to intervene in the parasite transmission and act against the transmission of Chagas disease.

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# CAPÍTULO 2

## Article

**Fungi pathogenicity to *Rhodnius prolixus* and potential for managing Chagas disease**

Aline Rafaela M. Garcia[1]; Adriana Rocha[1], Camila C. Moreira[1]; Silma L. Rocha[1], Alessandra A. Guarneri[2], Simon L. Elliot[1].

[1] Department of Entomology, Federal University of Viçosa, Campus Universitário, CEP: 36570-000, Viçosa, MG, Brazil.

[2] Centro de Pesquisa René Rachou, Avenida Augusto de Lima, 1715, CEP 30190-002, Belo Horizonte, MG, Brazil

## Introduction

Triatominae (Hemiptera: Reduviidae) are vectors of the flagellate protozoan *Trypanosoma cruzi*, which is the causal agent of Chagas' disease in Central and South America. This disease has a considerable medical and socioeconomic impact (Dias *et al*. 2002), with an estimated 8 to 12 million people infected with *T. cruzi* and 28 to 75 million individuals at risk of infection (Guhl & Lazdins-Helds 2007). More than 130 triatomine species can potentially be vector of *T. cruzi*. Of the 52 described species in Brazil, five are considered to be of epidemiological importance because of colonization in homes, and the other 47 species are wild and maintain the parasitic cycle only in wild mammals (Costa & Lorenzo 2009; Coura & Pinto Dias 2009).

Interventions to control triatomine vectors are based on their control using insecticides, particularly pyrethroids. These are usually applied to the internal walls of houses and to outhouses where foci of triatomines might be found (Coura & Pinto Dias 2009). However, insecticide resistance has been detected in some parts of South America, associated with ineffective treatments of deltamethrin against one the most important vectors *Triatoma infestans* (Orihuela *et al*. 2008).

Isolates of entomopathogenic fungi have been reported to be highly infective to triatomines and potentially represent an alternative tool for controlling of Chagas' disease vectors. Strains of *Metarhizium anisopliae*, *M. flavoviride* var. *pemphigi*, *M. robertsii*, *Isaria cateniannulata* and *Beauveria bassiana* can be highly infective to different life stages of *Rhodnius prolixus* and *T. infestans* under laboratory conditions (Romaña & Fargues 1987; Romaña & Fargues 1992; Luz *et al*. 1998; Rocha & Luz 2011). One *Beauveria bassiana* strain was equally infectious in populations of pyrethroid-resistant and susceptible *T. infestans*, with moderate rates of mortality in

preliminary experiments in rural village houses, demonstrating its potential for use in integrated vector and insecticide resistance management (Pedrini *et al*. 2009).

Selection of entomopathogenic fungi for use against insect pests require consideration of factors such as the pathogen's specificity, dose, the host and environmental factors (Marti *et al*. 2005). In applications of entomopathogenic fungi, the number of spores applied must be sufficient to ensure that the insect will come into contact with, and be infected by the inoculum. A highly virulent pathogen may require fewer propagules to cause the disease. However, isolates with good persistence are more likely to come into contact with the target insect and so infect it (Inglis *et al*. 2001). It is possible to obtain reasonably precise estimations of median lethal concentrations or doses ($LC_{50}$ or $LD_{50}$) from laboratory assays (Goettel & Inglis 1997). Under field and semi-field conditions, preliminary tests with an oil-formulation of *B. bassiana* in Central Brazil led to a reduction in *T. sordida* in peridomestic areas (Luz *et al*. 2004). Meanwhile, in the laboratory, the use of a formulation with diatomaceous earth gave 80-100% mortality in *T. infestans* after 5 and 10 days with a commercial *B. bassiana* strain (Forlani *et al*. 2011; Luz *et al*. 2012). Thus, it appears to be feasible, given the right isolate and formulation, to infect triatomines in the field.

The performance of fungi as biocontrol agents depends on environmental conditions as well as the behavioral response of the insect targeted (Elliot *et al*. 2002; Thomas & Blanford 2003). In this context, temperature could affect different biological parameters of triatomines (Luz *et al*. 1999; Guarneri *et al*. 2003) and also the development of entomopathogenic fungi inside the host (Thomas & Read 2007). Thus, it is of interest, in developing a fungal biocontrol agent, to have an understanding of how temperature affects the disease process.

A final consideration, and one of crucial importance for arthropod vectors, is that the aim of biocontrol is not primarily to exterminate or even control the insect

populations but to manage the disease in the human host, perhaps by blocking parasites transmission (Blanford *et al*. 2005; Scholte *et al*. 2005; Thomas & Read 2007). Thus, our first objective of the current study is to test the pathogenicity and virulence entomopathogenic fungi isolates for the control of Chagas disease. For this, we conducted three experiments: the first was a test of pathogenicity, aimed at assessing the infective potential of 10 isolates of *M. anisopliae* e *B. bassiana*, and their effect on survival of *R. prolixus*. The second was a dose-response experiment, to evaluate five different concentrations of two strains selected in the first test. In the third experiment, we aimed to investigate the effect of temperature on survival of insects infected by fungi.

## Material and Methods

### Insects

*Rhodnius prolixus* were obtained from a colony maintained at the Laboratory of Triatomine and Epidemiology of Chagas Disease (LATEC) of the René Rachou Research Center (CPqRR), Oswaldo Cruz Foundation in the State of Minas Gerais, Brazil. First instar nymphs, with 2-3 days old and starved, were used for pathogenicity bioassays. For the dose-response and temperature bioassays, first instar nymphs were blood-fed on chickens or mice and as were second instar nymphs within 3-4 days post-moult. During the bioassays the insects were not fed.

*Rhodnius prolixus* were reared under controlled conditions of temperature (26 ± 1°C) and relative humidity (65 ± 10%). Insects were exposed to a natural cycle of illumination and allowed to feed weekly on chicken or mice. All experiments using live animals were performed in accordance to FIOCRUZ guidelines on animal experimentation and were approved by the Committee of Ethics of Animal Use-FIOCRUZ (L-058/08).

### Production of spores and preparation of inoculum

We used 10 isolates of *M. anisopliae* (Metsch.) and *B. bassiana* (Bals.). Seven of these (C66A, J54A, J60A, L60A, C76B, S71B, L46C) were obtained from soil samples from coffee crops in Viçosa, using live baiting with larvae of *Tenebrio molitor* (Coleoptera: Tenebrionidae) (Moreira in prep.). Two isolates were kindly provided by the Federal Rural University of Pernambuco-Brazil, URPE-11 isolated from *Mahanarva posticata* (Hemiptera: Cercopidae) and URPE-18 obtained from soil. Isolate ENT-1 was from an unidentified coleopteran host from the Mata of Paraíso, Viçosa (Table 1).

All fungi were revitalized in *T. molitor* larvae and isolated on Petri dishes containing PDA (20% Potato, 2% Dextrose and 1.5% Agar) medium and incubated at 25 ± 1°C. Suspensions in sterile distilled water solution containing 0.01% of Tween 20® were prepared for each isolate and inoculated on rice. The rice was autoclaved for 15 min at 120ºC in polypropylene bags. Once cooled, a 2 ml aliquot of conidia suspension was added to 200g of the boiled rice. The bags were maintained in incubator at 25 ± 1°C for ten days to promote sporulation. Rice with spores was placed in Falcon tubes with sterile distilled water containing 0.01% Tween 20®. Suspensions were then agitated to release spores by vortexing for 3 minutes and were filtered through sterile gauze. The suspension concentrations were adjusted and standardized using a Neubauer hemocytometer and were used immediately after preparation.

To evaluate conidia viability, 100 µl of the main suspensions were placed on three Petri dishes containing PDA medium for each isolate and incubated at 25 ± 1°C. After 20 h, viability was assessed by checking 100 conidia for germination with the aid of an optical microscope at 400x.

Pathogenicity bioassay and screening of isolates

A randomized block design was used with twelve treatments and eight replications each. The twelve treatments were the ten isolates above and two controls: one received water and Tween 20® to check for cross-contamination and the other untreated filter paper to check for infection of the insects prior to experimental set-up. We placed five nymphs of first instar *R. prolixus* with 2-3 day old and starvation in each treatment. The nymphs were exposed to filter paper treated with 0.2 ml of fungal suspension at $1x10^8$ spores/ml and were placed in Petri dishes (60x15mm). These were sealed with plastic film to maintain humidity and were maintained at 25 ± 2°C, relative humidity of 80 ± 5% and 12 h photophase. After 48 h of exposure, the insects were

transferred to new dishes with untreated but humid filter paper. Nymphs were not fed during the assays and they remained in the dishes until they had all died.

Dead insects were placed in humid chambers to promote fungal development and sporulation to confirm if death was caused by fungal infection. These chambers were kept in an incubator at 25 ± 1°C. After two days, they were inspected under a stereomicroscope (40x) for external fungal growth and fungal identification. Dead insects with visible fungal growth on their body surface were considered to have died as a result of fungal infection.

Concentration-mortality bioassays

For this experiment, we chose two isolates to expose the nymphs of *R. prolixus* to five concentrations of conidia: $1x10^3$, $2x10^4$, $4x10^5$, $8x10^6$, $1.6x10^8$ spores/ml. The isolates URPE-11 and ENT-1 were chosen based on their infectivity to *R. prolixus* and high level of sporulation on rice at 10 days (Fig. 4). A dilution series was prepared in sterile distilled water solution containing 0.01% of Tween 20® and conidia counts were performed using a Neubauer hemocytometer for both isolates. A randomized block design was used with twelve treatments and six replicates for each treatment. We used five second instar nymphs of *R. prolixus* at 3-4 day post-moult for each replicate. Inoculation was as described above.

Effect of temperature on fungi pathogenicity

The two isolates URPE-11 and ENT-1, at concentrations $1x10^3$ and $2x10^4$ spores/ml respectively were used to expose *R. prolixus* to four constant temperature regimes 21, 23, 27 and 30°C. Inoculation was done above. The concentrations were selected as being borderline concentrations (based upon the results of the dose-response assay) to allow effects of temperature to be observed. We used three treatments for

each temperature and six replicates. For each replicate we used five second instar nymphs at 3-4 days post-moult. Nymphs were not fed during the assays.

**Data analysis**

Survival analyses were conducted using R software version 2.15.0 (R Development Core 2008) to all three experiments. We used Weibull distribution and the data was compared by comparison of means, means were considered to be statistically different at $P < 0.05$. Comparison between model was by one-way analysis of variance (ANOVA) and $\chi 2$ (chi-square test). Data were censored when the nymphs died before the end of the experiments.

## Results

Pathogenicity bioassay and screening of isolates

The mortality of the first instar nymphs of *R. prolixus* was 100% at eight days post-inoculation for all 10 fungal isolates tested (Figure 1), with $LT_{50}$ varying from 3.7 to 5.3 days. Survival times of the two control groups were statistically indistinguishable ($\chi^2_{[10]=}$15.987, $P$=0.06, $P$=0.09) and were considerably longer than the infected insect survival times (7.98±0.09, $\chi^2_{[09]}$=27.877, $P$<0.001), with only 5% of control insects dying by day 17.

Comparisons among models showed that there were three groupings of isolates for which nymph survival times were statistically indistinguishable ($P$>0.05). The group that killed insects most quickly ($LT_{50}$ 3.85±0.02 days) comprised *M. anisopliae* isolates C66A, L60A and L54A. The second group of isolates had an $LT_{50}$ of 4.02±0.01 days, slower than the first group ($\chi^2_{[09]}$=27.877, $P$<0.001), and comprised *M. anisopliae* isolates J60A, URPE-11 and *B. bassiana* isolate URPE-18 ($\chi^2_{[14]}$=13.339, $P$=0.42). This group in turn killed insects faster than the third grouping, of *B. bassiana* isolates ($LT_{50}$ 4.79±0.01 days, $\chi^2_{[09]}$=27.877, $P$<0.001).

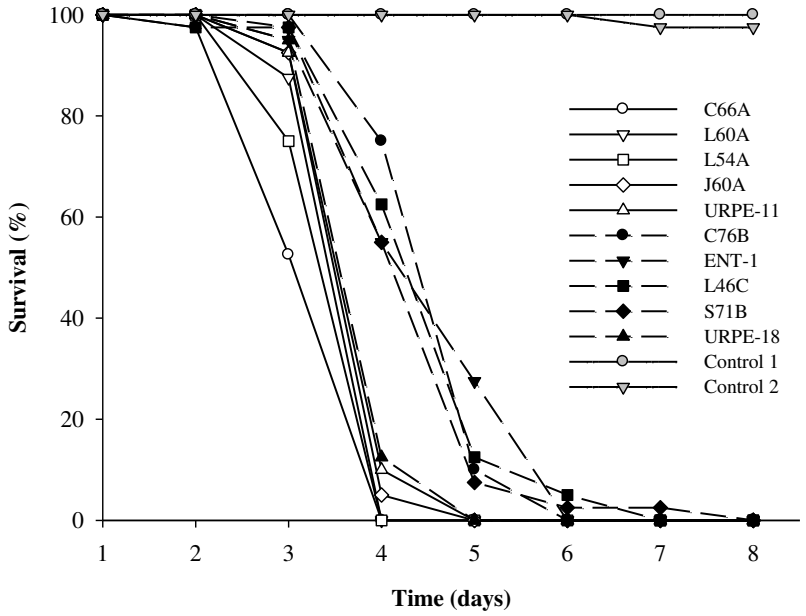


**Figure 1**. Survival of *Rhodnius prolixus* first instar nymphs infected with the fungi *Metarhizium anisopliae* (C66A, L54A, J60A, L60A, URPE-11) and *Beauveria bassiana* (C76B, ENT-1, L46C, S71B, URPE-18). Control 1 (Filter paper no treated) and Control 2 (Water + Tween 20).

Concentration-mortality bioassays

Second instar *R. prolixus* nymphs were inoculated with five concentrations of *M. anisopliae* URPE-11 and *B. bassiana* ENT-1 and were monitored for 20 days. At all but the lowest doses, *M. anisopliae* isolate URPE-11 caused 100% mortality by 7 days (Fig. 2A). Mean survival times were 3.96±0.23 (mean±SE), 4.4±0.23, 5.13±0.23, 6.06±0.23 and 9.96±0.24 days at concentrations $1.6x10^8$, $8x10^6$, $4x10^5$, $2x10^4$ and $1x10^3$ spores/ml respectively. All of these survival times were significantly different from one another and from the controls ($\chi^2_{[10]}$=23.209 for $P$<0.01) and all were significantly shorter than untreated controls ($P$<0.0001).

With the exception of the two lowest doses, *B. bassiana* isolate ENT-1 caused 100% mortality by 10 days (Fig. 2B). Mean survival times were 5.8±0.23 (mean±SE), 6.1±0.23, 7.53±0.23, 10.06±0.23 and 13.46±0.23 days for concentrations $1.6x10^8$, $8x10^6$, $4x10^5$, $2x10^4$ and $1x10^3$ spores/ml respectively. Survival times at the three higher doses were statistically indistinguishable from one another ($\chi^2_{[10]}$=15.987, $P$>0.17) but survival times of this cluster and the two lowest doses were significantly different from one another and from the controls ($\chi^2_{[10]}$=29.588 for $P$<0.001)

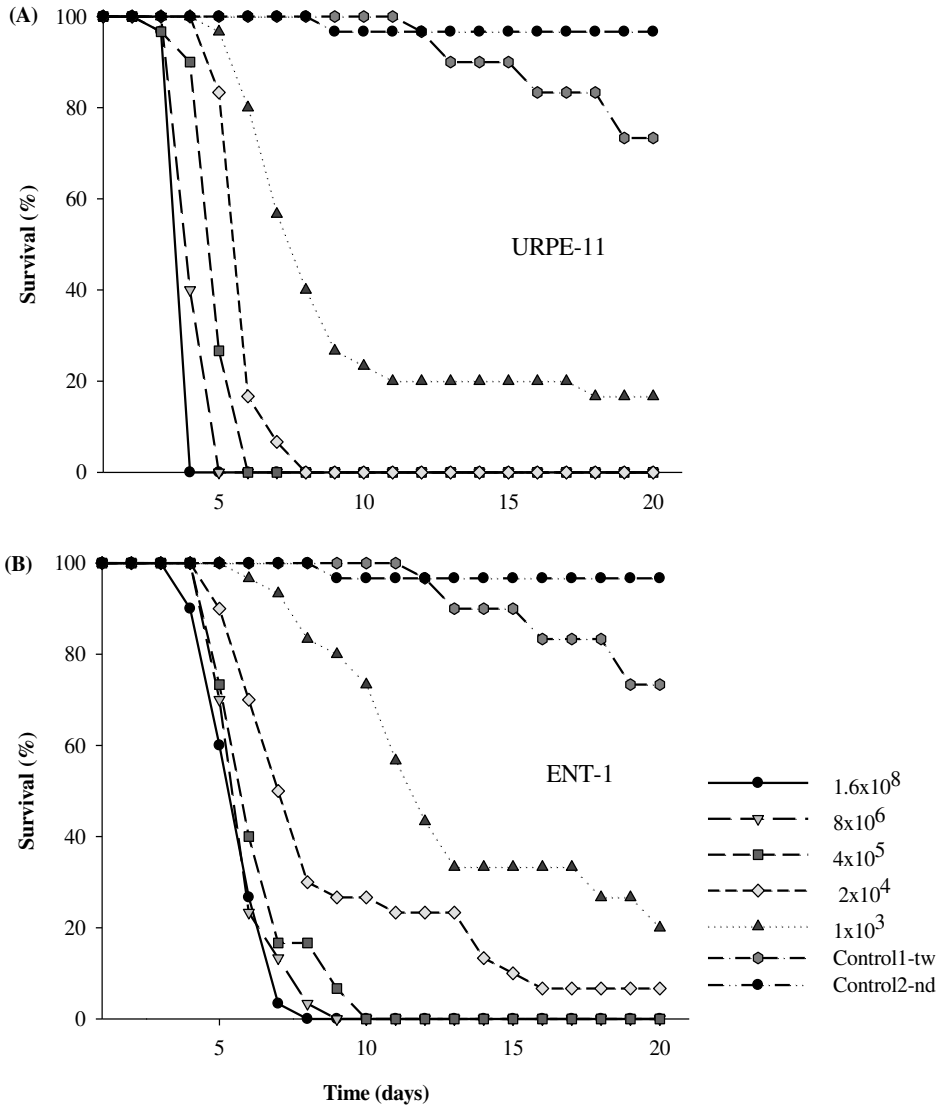


**Figure 2.** Survival of *Rhodnius prolixus* second instar nymphs inoculated with different concentrations ($1.6x10^8$, $8x10^6$, $4x10^5$, $2x10^4$ and $1x10^3$ spores/ml) of **(A)** *Metarhizium anisopliae* URPE-11 and **(B)** *Beauveria bassiana* ENT-1.

Effect of temperature on fungi pathogenicity

For insects infected with *M. anisopliae* URPE-11, survival times generally decreased with increasing temperatures. At 30°C, mean survival time was 7.2±0.05. This was shorter than the survival time at 27 and 23°C (11.43±0.06 and 11.03±0.06 days respectively; $\chi^2_{[6]}$=22.458 and $\chi^2_{[6]}$=22.458 respectively, both $P$<0.001) and mortality was 100% at 9 days. There was no difference in survival of insects at 27 and 23°C ($\chi^2_{[6]}$=5.348, $P$=0.46). The survival at 27 and 23ºC were, in turn, shorter than survival at 21ºC (13.83±0.08 days; $\chi^2_{[6]}$=22.458, $P$<0.001; $\chi^2_{[6]}$=16.812, $P$<0.01 respectively) with only 50% of mortality of insects in 15 days (Fig 3A). All of these survival times were significantly lower than controls ($P$<0.0001).

For insects infected with *B. bassiana* ENT-1 survival times also generally decrease with increasing temperatures. At 30 and 23°C the mean survival time were statistically indistinguishable from one another (7.16±0.05 and 7.63±0.05 days, respectively; $\chi^2_{[6]}$=7.841, $P$=0.262) and mortality was 100% at 10 days (Fig 3B). These times were shorter than and statistically different from survival time at 21 and 27ºC (10.4±0.03 and 11.6±0.06 days respectively; $\chi^2_{[6]}$=22.458 and $\chi^2_{[6]}$=22.458 respectively, both $P$<0.001). At 27ºC the survival was shorter than the survival time at 21ºC ($\chi^2_{[6]}$=22.458, $P$<0.001). All of these survival times were significantly lower than controls ($P$<0.0001).

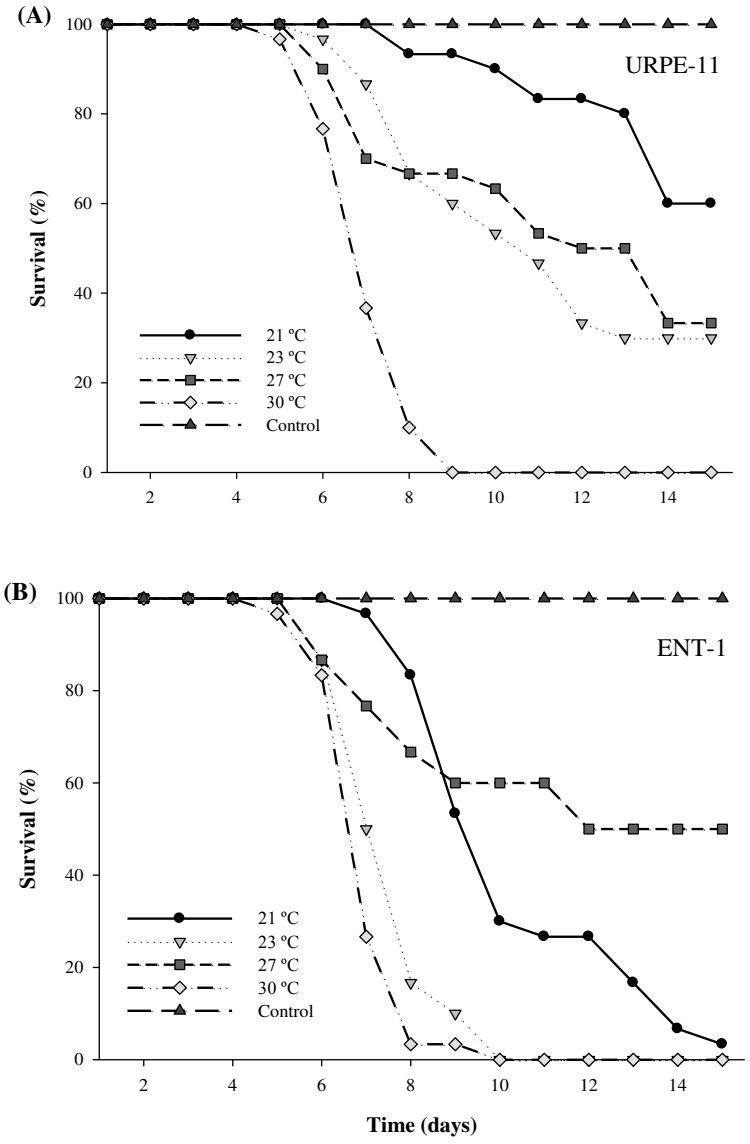


**Figure 3.** Survival of *Rhodnius prolixus* second instar nymphs infected with **(A)** *Metarhizium anisopliae* URPE-11 and **(B)** *Beauveria bassiana* ENT-1 kept at four constant temperature regimes 21, 23, 27 and 30°C.

## Discussion

All of the isolates tested were shown to be pathogenic to *R. prolixus*, in line with previous studies (Romaña & Fargues 1992; Luz *et al*. 1998). The species *B. bassiana* and *M. anisopliae* have a broad host range, spanning numerous orders within the Arthropoda (Hajek & St. Leger 1994), although individual isolates can be more restricted. They also can be pathogenic to vectors of parasites such as *T. infestans*, *T. sordida* and *R. prolixus* (Luz *et al*. 1998; Luz *et al*. 2004; Marti *et al*. 2005).

The impact of the infection on survival of nymphs was fairly similar among groupings of isolates, although there was higher virulence of *M. anisopliae* (group 1 - C66A, L54A and L60A) compared to group 2: two *M. anisopliae* and one *B. bassiana* (URPE-11, J60A and URPE-18) and group 3 of *B. bassiana* isolates (L46C, C76B, S71B and ENT-1). Given infective isolates, the efficiency of mass production, feasibility of long-term storage and operational criteria are key in choosing an isolate for development (Thomas & Read 2007). Thus, it was noted that the isolates URPE-11 (*M. anisopliae*) and ENT-1 (*B. bassiana*) required less time to sporulate on solid rice compared to other isolates tested. Thus, these isolates were selected for the bioassays for dose and temperature.

An important consideration in selecting a strain is its virulence, usually expressed as a median lethal dose or $LD_{50}$ (Inglis *et al*. 2001) which is a measure of how much of a pathogen can cause disease in the target insect. Here, we found that *M. anisopliae* isolate URPE-11 was more virulent against 2$^{nd}$ instar nymphs, including at the lowest concentration $2x10^2$ spores/ml. This isolate negatively affected survival from 4 days post-inoculation and caused 100% of mortality by 9 days. Meanwhile insects inoculated with *B. bassiana* ENT-1 recorded 100% mortality of insects on high concentration by 8 days. This comparison suggests that the *B. bassiana* isolate is less

virulent than the *M. anisopliae* isolate. According to some authors, pathogens do not necessarily need to kill the insect or to kill it rapidly, in order to achieve the management goals (Thomas & Read 2007).

Entomopathogens have the potential to influence behavior and fitness of the insects and may negatively affect diseases transmitted by vectors (Blanford *et al*. 2005) and this has been shown in studies that emphasize pre or sub-lethal effects to malaria and dengue vectors (Scholte *et al*. 2006; Howard *et al*. 2010; Blanford *et al*. 2011; Mnyone *et al*. 2011). For this reason, differences between isolates can be better investigated. The isolates (URPE-11 and ENT-1) although it was not more virulent among the isolates tested after initial screening, showed difference in virulence at different concentrations, moreover, greater sporulation in rice indicates an important aspect for the development of mycosinsecticide for mass production.

We tested isolates URPE-11 and ENT-1 in second instar nymphs at four fixed temperature regimes and found a significant reduction in survival times of these insects with increasing temperature. According to Schilman & Lazzari (2004) the mean preferred temperature for *R. prolixus* is 25.0ºC for males and 25.4ºC for females. When starved, these insects preferred slightly lower temperatures, approximately 24ºC, and this preference only changed with prolonged food deprivation (Schilman 1998). Adverse conditions of temperature, in the field, can affect the performance of these pathogens and affect the behavior of the hosts. In this context, isolates able to infect in adverse conditions of temperature are preferable.

In our study, insects inoculated with *B. bassiana* ENT-1 isolate had 100% mortality at low and high temperatures. Meanwhile, insects infected with *M. anisopliae* isolate URPE-11, at first sight more virulent, had lower mortalities at the lower temperature. This indicates that temperature can affect the virulence of these isolate. Maybe the origin of the isolates, explains the differences in virulence to insects at

different temperatures. The URPE-11 is origin from the northeast region of Brazil, and may be selected for high temperatures, explaining low virulence to insects at lower temperatures. Meanwhile ENT-1 was isolated of the host from Mata do Paraíso, Viçosa, Minas Gerais, where there is greater temperature fluctuation during the year, so the fungus may be more of a thermal generalist.

These results may have some implications for control of Chagas disease vectors under field conditions. It seems, then, that ENT-1 isolate could be a better candidate for a bioinsecticide for management of Chagas disease vectors. Further work is needed on its pathogenicity in other triatomine vectors, in insecticide-resistant vectors and formulation. Perhaps, though, it is more important as a next step, to investigate sublethal effects of fungal infection on these vectors and in particular their capacity to transmit parasites.

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## CONCLUSÕES GERAIS

- Todos os isolados testados mostraram serem patogênicos para *Rhodnius prolixus*. Os isolados de *Metarhizium anisopliae* demonstraram ser mais virulentos às ninfas de primeiro instar em relação aos isolados de *Beauveria bassiana*;
- Os isolados ENT-1 e URPE-11 apresentaram maior esporulação em arroz (critérios importante para a produção em massa de esporos);
- O isolado URPE-11 (*M. anisopliae*) mostrou ser mais virulento que ENT-1 (*B. bassiana*) inclusive na menor concentração.
- Ninfas de segundo instar infectados com URPE-11 e ENT-1 tiveram redução significativa no tempo de sobrevivência com o aumento da temperatura.
- Obtivemos 100% de mortalidade dos insetos inoculados com *B. bassiana* em altas e baixas temperaturas.
- Insetos inoculados com o isolado URPE-11 (*M. anisopliae*), tiveram mortalidade mais baixas na temperatura mais baixa, comparado a ENT-1 (*B. bassiana*), indicando que a temperatura pode afetar a virulência destes isolados.
- O isolado ENT-1 demonstra ser um bom candidato a bioinseticida para o tratamento da doença de Chagas em condições adversas de temperaturas.
- Faz-se necessárias investigações sobre a patogenicidade a outras espécies de triatomíneos, espécies resistentes a inseticidas e efeitos subletais da infecção fúngica na capacidade de transmitir a doença.

# ANEXOS

**Figure 4**. Isolates cultivated on rice for sporulation **(A)** *B. bassiana* and **(B)** *M. anisopliae*. Here, the photography take at seven days after inoculated to *Metarhizium* and six days to *Beauveria*.

**Figure 5**. Sporulation of *Beauveria bassiana* on *Rhodnius prolixus* – Image: Harry Evans

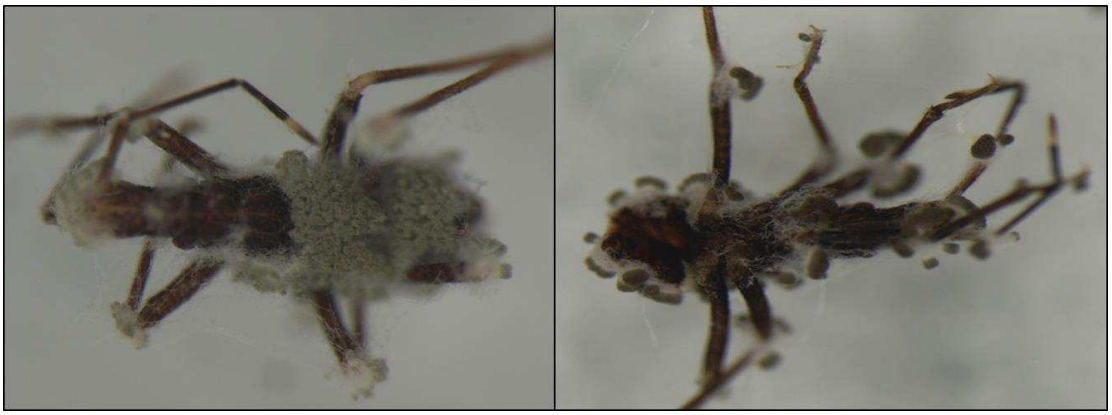

**Figure 6**. Sporulation of *Metarhizium anisopliae* on *Rhodnius prolixus* – Image: Harry Evans

**Table 1: Origen of fungi isolates**

| Isolate | Host/substrate | Geographic/origin |
|---|---|---|
| *Metarhizium anisopliae* | | |
| L54A | Soil/*Tenebrio molitor* | Minas Gerais/Brazil |
| L60A | Soil/*Tenebrio molitor* | Minas Gerais/Brazil |
| C66A | Soil/*Tenebrio molitor* | Minas Gerais/Brazil |
| J60A | Soil/*Tenebrio molitor* | Minas Gerais/Brazil |
| URPE-11 | Hemiptera: *Mahanarva posticata* | Recife/Pernambuco/Brazil |
| *Beauveria bassiana* | | |
| C76B | Soil/*Tenebrio molitor* | Minas Gerais/Brazil |
| S71B | Soil/*Tenebrio molitor* | Minas Gerais/Brazil |
| L46C | Soil/*Tenebrio molitor* | Minas Gerais/Brazil |
| Ent-1 | Coleoptera | Minas Gerais/Brazil |
| URPE-18 | Soil | Cabo/Pernambuco/Brazil |