PRISCILLA LARA FARIA

SCREENING FUNGAL ISOLATES FOR BIOLOGICAL CONTROL OF CULICID VECTORS BASED ON FORMATION OF APPRESSORIA

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: 27 de julho de 2011.

_____
Prof. Gustavo Ferreira Martins
(Coorientador)

_____
Dra. Alessandra Aparecida Guarneri

_____
Prof. Simon Luke Elliot
(Orientador)

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RESUMO

FARIA, Priscilla Lara, M. Sc., Universidade Federal de Viçosa, julho de 2011. **Rastreamento de isolados fúngicos para o controle biológico de culicídeos vetores baseado na formação de apressório.** Orientador: Simon Luke Elliot. Coorientadores: Gustavo Ferreira Martins e Angelo Pallini.

O controle de doenças transmitidas por insetos vetores pode ser realizado através do controle do agente etiológico da doença ou do controle do vetor da doença. Entretanto, devido às dificuldades na produção de vacinas, à baixa eficácia dos compostos repelentes e aos problemas com inseticidas sintéticos, formas alternativas no combate aos vetores vêm sendo divulgadas. Entre as alternativas, o controle biológico com uso de fungos entomopatogênicos vem se destacando, seja visando o rápido extermínio do vetor, controlando diretamente sua população, ou ocasionando efeitos subletais que podem influenciar na capacidade vetorial destes insetos. A especificidade destes entomopatógenos aos seus hospedeiros ocorre, provavelmente, através de processos de reconhecimento que podem estar ligados à composição da epicutícula de seu inseto-alvo, demonstrando uma resposta diferente a determinados hospedeiros. No presente trabalho estudou-se uma técnica de rastreamento de isolados dos fungos *Beauveria bassiana* e *Metarhizium anisopliae* utilizando a cutícula das asas dos mosquitos vetores *Aedes aegypti* e *Anopheles aquasalis*. Essa técnica consistiu em inocular uma suspensão de $1x10^5$ conídios ml^{-1} nas asas dos mosquitos e verificar, após 30 horas, a germinação e desenvolvimento de apressórios, buscando selecionar isolados que formassem esta estrutura de infecção em ambos os mosquitos. Confirmando a funcionalidade da técnica, nós verificamos que de 83 isolados testados em *Ae. aegypti,* 3 não germinaram e 67 germinaram e formaram apressórios. Em *An. aquasalis*, de 40 isolados testados, todos germinaram e destes, 32 germinaram e formaram apressórios. De 40 isolados testados em *Ae. aegypti* e *An. aquasalis*, 32 formaram apressórios em ambos, corroborando com estudos que relatam a seletividade dos fungos e a influência de

compostos cuticulares no reconhecimento do hospedeiro. Estes fungos selecionados serão investigados para o controle biológico de mosquitos.

ABSTRACT

FARIA, Priscilla Lara, M. Sc., Universidade Federal de Viçosa, July, 2011. **Screening fungal isolates for biological control of culicid vectors based on formation of appressoria.** Adviser: Simon Luke Elliot. Co-Advisers: Gustavo Ferreira Martins and Angelo Pallini.

The control of diseases transmitted by insect vectors may be performed by control of the etiological agent of the disease or by vector control. However due to difficulties with vaccine production, limited effectiveness of repellent compounds and the problems with synthetic insecticides, alternative means of vector control are of interest. Among these, biological control using entomopathogenic fungi has generated interest, whether for vector population control due to mortality, or through sublethal effects that can influence vectorial capacity. The host specificity of entomopathogenic fungi is likely to occur through recognition processes linked to the composition of the epicuticle of the insect target, showing different responses to different hosts. Here, we studied a technique for isolate screening of *Beauveria bassiana* and *Metarhizium anisopliae* using the wing cuticle of the mosquito vectors *Aedes aegypti* and *Anopheles aquasalis*. This technique consists of inoculating a suspension of $1x10^5$ conidia/ml^{-1} on the wings of mosquitoes and determining, after 30 hours, conidial germination and appressorium development (this being necessary for infection), with the aim of selecting isolates that formed appressoria in both mosquito species. Confirming the functionality of the technique, we found that of 83 isolates tested in *Ae. aegypti*, 3 did not germinate, while 67 germinated and formed appressoria. In *An. aquasalis*, 40 isolates was tested and all germinated; of these, 32 germinated and formed appressoria. Of 40 isolates tested in both mosquitoes, 32 formed appressoria in both, in line with studies that report the selectivity of the fungi and the influence of cuticular compounds in recognition of the host. These selected fungi are being investigated for biological control of mosquitoes.

GENERAL INTRODUCTION

Every year, millions of people contract diseases transmitted by insect vectors. These diseases have a large impact on social and economic activities, especially in tropical countries (Zaim & Guillet, 2002). Control programs have always prioritized arthropod vector control. However, financial and management problems, together with misuse of insecticides have often led to the failure of these programs (Gubler, 1998).

Among the insect vectors of greatest importance, the genera *Aedes* and *Anopheles* stand out, being responsible respectively for the transmission of arboviruses such as Chikungunya fever (WHO, 2008), dengue and yellow fever, and the malarial parasite *Plasmodium* (Hemingway & Ranson, 2000; WHO, 2009; 2011). Emergence and resurgence of these arboviruses (Gubler, 1996; 2002) and the continued prevalence of malaria (Scholte *et al.* 2003) highlight the need for greater efforts to control these mosquitoes.

Options for vectorborne disease control include the control of etiological agents through vaccination or control of the vectors. For some arboviruses, however, there are no vaccines currently available, so vector control continues to be extremely valuable. Therefore, the search for sustainable ways of controlling insect vectors of human diseases has high value, as these are key factor of control components (Zaim & Guillet, 2002). Options for vector control include the direct control of mosquito populations and indirect control via pre-death effects (called sublethal effects). Direct control is fundamentally based on the use of insecticides, with a view to immediate death of the insect. The advantage of this method is that it prevents mosquito breeding, in addition to preventing new blood meals and thus, prevents transmission of the etiological agent of disease.

Although insecticides are important tools for insect vector control (Hemingway & Ranson, 2000; Scholte *et al.*, 2004; Thomas & Read, 2007) and are considered one of the cheapest and most effective methods to control mosquitoes (Read *et al.*, 2009) and other insect vectors (Zaim & Guillet, 2002), their indiscriminate use, besides causing, environmental contamination and health problems for humans and other animals, induce selection for insect resistance (Lacey & Undeen, 1986; Hemingway & Ranson, 2000; Lacey *et al.,*

2001; Shiff, 2002; Hargreaves *et al.*, 2003). A further factor is that these chemicals are derived from exhaustible sources such as petroleum (Messias, 1989). Thus, there is a need for new control tools (Zaim & Guillet, 2002; Farenhorst *et al*., 2008; Howard *et al*., 2010a,b) that do not harm the environment and are safe for use (Vega *et al.,* 2009), also that are economically and ecologically viable (Valadares-Inglis *et al*., 1998).

Indirect control, via sublethal effects, does not require immediate insect death and has a slower action. It is possible that this means of control decreases the selection pressure for resistance, where it allows insect reproduction (Thomas & Read, 2007). It may thus be interesting to develop interventions that act sublethally on the insect's vectorial capacity. Decreases in insect longevity, in blood feeding success, in fecundity and in vector competence, are among the sublethal effects sought.

Within this context, biological control is an alternative to be used against these vectors (Scholte *et al.*, 2004; Mohanty *et al.*, 2008; Blanford *et al.*, 2009). The advantages of these agents are intrinsically linked to its action and production mode, limited side effects, longer-lasting control and are non-toxic (Alves, 1998). In addition to these factors, may also be motivated by potential for sublethal effects such as reducing mosquito longevity (Blanford *et al.,* 2005; Scholte *et al.,* 2003; 2005; 2007; de Paula *et al*., 2008; Kannan *et al*., 2008; Mohanty *et al.,* 2008; Leles *et al.*, 2010; Mnyone *et al*., 2011), blood feeding success (Scholte *et al*., 2006; Howard *et al*., 2010a), fecundity (Scholte *et al*., 2006) and vector competence (Blanford *et al*., 2005).

Another important factor is that an entomopathogenic fungus does not need to be ingested to infect its host as it can infect through the cuticle (Pedrini *et al*., 2007; Scholte *et al*., 2007) via mechanical pressure and enzymatic action. However, for infection to occur there must be adhesion and formation of infection structures (Charnley, 1984), which only occurs when there is host recognition, indicative of specificity (Charnley, 1984; St. Leger *et al*., 1990a). The surface structure and chemical composition of the host cuticle seems to influence this process (Pedrini *et al*., 2007). Specificity may be related to mechanisms of recognition associated with the composition of the host epicuticle, since these components are extremely heterogeneous. These variations may lead to different pathogen responses to particular insects (St.

Leger *et al.*, 1990a; 1996a). Although the chemical interaction between fungi and host during adhesion is poorly explained (Boucias *et al*., 1988, Yaginuma *et al*., 2006), it is known that a fungus' virulence is correlated with its ability to infect the host cuticle (Yaginuma *et al*., 2006). This difference in the response of a pathogen can be used for selection of isolates (Wang & St. Leger, 2005).

Traditionally, studies aimed at selecting entomopathogenic fungi are conducted by inoculating the fungus in live insects, observing the mortality caused in their host and selecting the isolate that killed most quickly (Neves & Hirose, 2005; Almeida *et al*., 1997; Chernaki-Leffer *et al*., 2007). These experiments can require a large investment of time and resources. In mosquitoes, isolates which cause high mortality (considered highly virulent) are selected for further studies, discarding less virulent fungi, as seen in the work of De Paula *et al*. (2008). However, these less virulent fungi may be used to produce sublethal effects that can be useful in insect vector control.

We therefore tested a technique for screening of fungal isolates that should facilitate and expedite the screening of fungi able to infect mosquitoes, regardless of their virulence. Specifically, we investigated the development of infection structures of Brazilian isolates of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* on the cuticle of wings of the culicid vectors *Aedes aegypti* and *Anopheles aquasalis*.

Work with grasshoppers suggests that the wings of these insects consist essentially of two layers of cuticle, without fat body and with little other material (Uvarov, 1966 *apud* Jarrold *et al.,* 2007) and the epicuticular lipid composition is similar to that found elsewhere in the insect's cuticle (Lockey and Oraha, 1990). However, for the mosquitoes, the wing cuticle composition may not be iqual to the grasshoppers, but allows the fungal growth. Furthermore, the choice of the wings for screening is justified because they are comparatively easy to observe with optical microscopy due to their transparency, facilitating the visualization of the fungus development. Both fungi used target a wide range of insects at the species level (Thomas & Read, 2007), though there is specificity at the strain level. They have already been tested against eggs (Luz *et al*., 2008), larvae and adults of mosquitoes (Scholte *et al*., 2003; 2004). Furthermore, there are products formulated from these fungi that are already marketed in Brazil to

control insect pests. We included commercial biopesticide products based on the fungi *B. bassiana* and *M. anisopliae* in our tests.

We adapted the technique previously used on locust and cicada wings (Wang & St. Leger, 2005), and this is the first time it has been tested for mosquito vectors. This alternative can be used to screen fungal isolates when there is a limited availability of insects for mortality tests, or if the aim is to find isolates that can infect more than one host. Here, we aim to screen isolates against representatives of the genera *Aedes* and *Anopheles*.

1. BIBLIOGRAPHIC REVIEW

1.1 *Aedes* and *Anopheles*

There are approximately 3,300 species of mosquitoes (Culicidae), distributed in 41 genera and three subfamilies, Toxorhynchitinae, Anophelinae and Culicinae (Service, 2004). Mosquitoes are distributed around the world, with the exception of some permafrost regions (Forattini, 2002a).

The genus *Aedes* is responsible for transmitting *Flavivirus* (Flaviviridae) that causes yellow fever and dengue (Luz *et al.*, 2008), in addition to *Alphavirus* (Togaviridae) that causes Chikungunya fever (WHO, 2008). The requirement of these viruses for arthropod blood to complete their life cycle means that they are also known as arboviruses (Mackenzie *et al.*, 2004). *Aedes* can be found widely distributed between latitudes 35° N and 35° S (Gibbons & Vaughan, 2002), in tropical and subtropical regions. It is highly anthropophilic (Harrington *et al*., 2001), considered extremely important because female feeds on blood of more than one individual during a single gonotrophic cycle (Harrington *et al.*, 2001; Mackenzie *et al*., 2004). Moreover, it has a preference for human blood (Harrington *et al*., 2001) which enables the transmission of disease to more than one person.

Around 2.5 billion people are now at risk from dengue and it is estimated that there are 50 million dengue infections worldwide every year (WHO, 2009). *Aedes aegypti* is considered the cause of most deaths and morbidity among human arthropod vectors (Harrington *et al.*, 2001), largely due to dengue hemorrhagic fever, which is more common after a secondary infection with the virus (Gibbons & Vaughan, 2002). Throughout the year, mosquitoes have the ability to reproduce using a wide variety of sites for oviposition. Females oviposit mainly above the waterline on damp surfaces. Egg hatch occurs when the eggs are immersed in water. Larvae develop in the aquatic environment until pupation, while the adult lives in the terrestrial environment (Becker *et al*., 2010a). Female mosquitoes acquire the virus when feeding on the blood of an infected person and virus incubation takes from 8 to 10 days (WHO, 2009). Once infected, the mosquito can transmit the virus for the rest of its life. In, addition, there may be transovarial transmission (Joshi *et al.*, 2002). In infected

humans, the virus circulates in the blood for two to seven days, corresponding to the period in which there are symptoms (WHO, 2009).

The development of cities, transportation and land use changes have been especially conducive to the emergence and resurgence of diseases. Additionally, natural factors may also contribute to the spread of these viruses, such as genetic changes in the viruses, host-vector relations and environmental factors (Murphy & Nathanson, 1994; Mackenzie *et al*., 2004).

Yellow fever is also associated with the mosquito *Ae. aegypti.* This is a viral hemorrhagic disease, endemic in tropical areas of Africa and Latin America, transmitted by infected mosquitoes. Incubation of virus in the body takes 3 to 6 days and at this time, the mosquito can become infected when taking the blood meal. Data estimate that there are 200,000 cases of yellow fever, causing 30,000 deaths worldwide each year. This is in spite of the availability of a vaccine that provides effective immunity against yellow fever within a week, protecting for 30-35 years or more (WHO, 2011).

Another disease that has caused concern is Chikungunya fever. More than 1.25 million people in India and south Asia were infected with the chikungunya virus from February to October 2006. Chikungunya is a viral disease, spread by mosquitoes, that causes fever and severe joint pain, but in most cases, symptoms are mild. It was described during an outbreak in southern Tanzania in 1952. Between four and eight days after infection, onset of illness occurs. This disease occurs in Africa, Asia and India and more recently, was reported for the first time in Europe. The resurgence and geographic spread of this disease emphasizes the need for sustainable measures to control its transmission (WHO, 2008).

Malaria is also a clear example of a not eradicated disease (Krogstad, 1996). The genus *Anopheles* is the principal vector of the parasite *Plasmodium,* the causative agent of malaria which is a major tropical parasitic disease, having worldwide distribution (Rebelo *et al*., 1997). The mode of reproduction of this mosquito is similar to *Aedes* in that it is the haematophagous female that transmits the disease. Two important ecological and epidemiological aspects in populations of some anophelines are their prevalence in the rainy season and peak biting at dusk and early evening hours (Xavier & Rebelo, 1999). Over the past 20 years, different species of *Anopheles* were considered as secondary

transmitters or potentially capable of transmitting malaria in the Brazilian Amazon region, however, *An. darlingi,* remains the primary vector. Infection by malaria has increased in recent decades, despite the efforts of governmental disease control services (Couto *et al*., 2010).

Anopheles aquasalis Curry 1932 (Da Silva *et al*., 2006) is considered a coastal vector (Zimmerman, 1992), being an important vector in these areas. Found in coastal regions, with tidal influence, this mosquito breeds in salt water, the salt apparently being of fundamental importance for its development (Forattini, 2002b). Haematophagous habits vary, but the females seem to be more zoophilic, but with some anthropophilicism when at high densities. In some regions, *An. aquasalis* is considered the main vector of malaria. In northeastern Brazil, it is considerably endophilic and anthropophilic. Associated with their density, longevity also appears to be an important factor in the vectorial capacity of this species (Forattini, 2002b).

1.2 Biological control and entomopathogenic fungi

A large number of compounds has been invented and marketed to control insect pests, beyond cultural controls (Hajek, 2004b). The control of pests by living organisms, referred to as biological control, is the use of predators, parasitoid, pathogens, antagonists or competitors to suppressing a pest population (Van Driesche & Bellows Jr, 1996; Hajek, 2004a).

In the biological control of mosquitoes, predators and entomopathogens have produced promising results (Scholte *et al.,* 2004). Predators such as fish (Legner, 1995) and copepods (Nam *et al*., 2005), and entomopathogenic fungi, bacteria (Lacey & Undeen, 1986, Chung *et al*. 2001; Seleena *et al*. 2001; De Deken *et al*., 2004; Romi *et al*., 2006; Majambere *et al*., 2007), nematodes (Kaya & Gaugler, 1993; Platzer, 2007) and protozoa (Chapman, 1974; Legner, 1995; Becnel & Johnson, 2000; Micieli *et al*., 2000) have all been investigated for biological control. Bacterial larvicides are now widely used.

The study of entomopathogens dates from the early 1800's, beginning with work on fungi pathogenic to silkworm (Vega *et al*., 2009), and tests of the fungus *Metarhizium anisopliae* against cockchafer. Since then much work has shown the potential for use of entomopathogenic fungi against pests (Gillespie

and Moorhouse, 1994) and infection process in other insects, on the most diverse orders, such as Coleoptera (Kershaw *et al*., 1999; Neves & Hirose, 2005; Yaginuma *et al*., 2006; Rangel *et al*., 2008; Skrobek *et al*., 2008), Lepidoptera (Kershaw *et al*., 1999; Skrobek *et al*., 2008), Orthoptera (Inglis *et al*., 1996; Kershaw *et al*., 1999; Donatti *et al.*, 2008).

Entomopathogenic fungi are found in soil environment and are natural enemies of insects and mites, playing a role in population regulation of these organisms (Pell *et al.,* 2010). Also, they can be found in plants tissue, growing endophytically (Quesada-Moraga *et al*., 2006; Akello *et al*., 2009; Gurulingappa *et al*., 2010). They are extremely diverse: it is believed that there are approximately 90 genera and over 750 species of the entomopathogenic importance, highlighting the Classes Oomycetes, Chytridiomycetes, Ascomycetes e Zygomycetes (Goettel *et al.* 2010).

The Ascomycetes include members of the Hypocreales that have interactions not only with arthropods, but plants and other fungi as well; however, no other group of Ascomycetes has so many associations with arthropods (Blackwell, 2010). Several hypocrealean fungi have been found to be pathogenic to *Aedes* (de Paula *et al*., 2008; Luz *et al*., 2008; Leles *et al*., 2010), *Anopheles* (Scholte *et al*., 2006; Farenhorst *et al*., 2008; Mohanty *et al*., 2008; Howard *et al*., 2010b; Mnyone *et al.,* 2010) and other mosquitoes (Alves *et al.,* 2002; Mohanty *et al*., 2008; Howard *et al*., 2010a; Luz *et al*., 2010). Among these are: *Metarhizium*, *Isaria* (*Paecilomyces*), *Lecanicillium* (Leles *et al*., 2010), *Fusarium* (Mohanty *et al*., 2008) and *Beauveria* (de Paula *et al*., 2008).

Some characteristics of entomopathogenic fungi are of great importance such as their specificity, relative ease of production and limited side effects. In addition, they do not contaminate the environment and are non-toxic to humans and other animals when selected and handled properly (Alves, 1998). They also have a wide variety of hosts, allowing the choice of more target-specific strains (Clarkson & Chanley, 1996) beyond the sublethal effects.

In this sense, sublethal effects such as reducing mosquito longevity (Blanford *et al.* 2005; Scholte*, et al.* 2004, 2005, 2007; de Paula *et al*., 2008; Kannan *et al*., 2008; Mohanty *et al.* 2008; Leles *et al.*, 2010; Mnyone *et al*., 2011), blood feeding success (Scholte *et al*., 2006; Howard *et al*., 2010a), that

might also be attributed to the action degradation of their tissues due to mycelial growth (Scholte *et al*., 2006). Fecundity is also reduced (Scholte *et al*., 2006), due to histological and cytological damage to the ovaries (Sikura *et al.*, 1972 *apud* Scholte *et al*., 2006) or lack of availability of resources for reproduction due to decreases in fat bodies (Arthurs & Tomas, 2000), while vector competence can also be reduced (Blanford *et al*., 2005), due to decreased numbers of bites, hindering the transmission of etiological agents of the disease. Thus, these sublethal effects are extremely important for disease control, since all interfere with disease transmission.

An important aspect of mosquito control with entomopathogenic fungi is that the fungi generally cause high mortality by day 14 after blood feeding. This can be related to the dengue, yellow fever and malaria cycle. For dengue and yellow fever, this infection will have less impact than for malaria (Scholte *et al*., 2007), since dengue virus has an incubation period of 8-10 days (WHO, 2009) and yellow fever virus has an incubation period of 3-6 days (WHO, 2011), while in malaria cycle, the sporozoites are present in the mosquito mouthparts by 14 day (Thomas & Read, 2007).

Furthermore, there is an increase in daily mortality of mosquitoes infected by both the fungus and *Plasmodium* and, at the same time; the number of mosquitoes with sporozoites in their mouthparts is reduced (Blanford *et al*., 2005). In addition to allowing reproduction of mosquitoes, there is reduced selection pressure, since it does not kill mosquitoes instantly. Thus, mosquitoes can breed and transmit their genes to future generations. This reduces the possibility of development of resistance to this form of control (Thomas & Read, 2007).

Before the 1940's, studies with entomopathogenic fungi were limited, given that *Metarhizium anisopliae* was being mass-produced by Krassilstchik and used against the sugar-beet weevil in field (Vega *et al.,* 2009). In the 1940's and 1950's, with the discovery and large-scale use of synthetic insecticides to control mosquitoes, biological control decreased and was largely supplanted (Becker *et al*., 2010b). The use of insecticides is an important tool (Hemingway & Ranson, 2000; Scholte *et al.*, 2004; Thomas & Read, 2007) and is considered one of the cheapest and most effective methods to control both insects that transmit malaria (Read *et al.*, 2009) and other vectors (Zaim & Guillet, 2002).

However, the role of synthetic chemical insecticides has been reconsidered after problems with their use, like resistance selection, and with its development (Lacey & Undeen, 1986; Hemingway & Ranson, 2000; Lacey *et al.,* 2001; Shiff, 2002; Hargreaves *et al.*, 2003). This has led to interest in entomopathogens as a means of pest control (Charnley, 1984).

1.3 Infection process of entomopathogenic fungi

For biological control, one of the major attributes of entomopathogenic fungi is their ability to infect the host through the cuticle, and their capacity to penetrate even the most sclerotic and normally resistant arthropod cuticle (Williams, 2003). The first step in fungal infection is the attachment of the conidia in the compatible host; this is the initial step of mycosis (Charnley, 1994). This step may be linked to the composition of the spore wall, where it is believed that there are determinants for the fixation of spores on the host cuticle. However, as with other fungi, it has been shown (Charnley, 1994) that conidial attachment of *Metarhizium anisopliae* and *Beauveria bassiana* to the host cuticle is nonspecific and passive, without the release or synthesis of adhesive material (Boucias *et al*., 1988). The interaction between conidia and host cuticle is not well understood (Charnley, 1994), but surface structure and chemical composition of the cuticle seems to influence this process, with chemical and topographical clues important in the recognition of a susceptible host (Pedrini *et al*., 2007).

The insects' cuticle is thin but its structure is complex formed by extra-cellular matrix composed of proteins, carbohydrates and lipids. It covers almost the entire surface of the insect, including foregut, hindgut and trachea. It is the outermost layer of the integument and it is formed by the solidification of the material secreted by epidermal cells (Gallo *et al*., 2002). It is divided into several layers: the epicuticle, the procuticle and the epidermis (Gullan and Cranston, 2005). The composition of the epicuticle seems to play a role in host recognition by fungi, since its components, in contrast to the procuticle, are extremely heterogeneous (Charnley, 1984). The procuticle consists mainly of proteins and chitin, but other materials may also be present, such as lipids and pigments, among others. The amount, organization and interaction between the

components will determine the general properties of the cuticle (Andersen, 1979). It represents a barrier to both chemical and biological insecticides, including acting as a physical barrier to the invading fungus (Hajek & St. Leger, 1994; Samuels & Paterson, 1995).

The germination of fungal conidia proceeds with the formation of a germ tube and appressorium or appressoria (Madelin *et al*., 1967), while enzymes are simultaneously produced to degrade the host cuticle (St. Leger *et al*., 1990b; 1996b). It is a complex interaction between insect host and fungal pathogen (Castrillo *et al.,* 2005), where an isolate of the same entomopathogenic fungus can exhibit different host specificities (McCoy, 1990) and respond in different ways (St. Leger *et al*., 1990a). Depending on the host, the production of enzymes may vary in the penetration phase, with variation in toxins during the post-penetration phase (vegetative growth within the host) and formation or not of infection structures. In the case of *M. anisopliae* and *B. bassiana*, the formation of the appressorium seems to be fundamental for establishment of infection and for the pathogenic relationship with the host (Clarkson and Charnley, 1996). Later, the appressoria form infection pegs in the epicuticle and penetrant hyphae invade the procuticle.

Penetration is effected by a combination of mechanical pressure and enzymatic degradation (Charnley, 1994). This step determines the success of parasitism in plants and animals. In plants, the infection structures are able to penetrate several types of cell walls (Mendgen *et al*., 1996). These morphological alterations suggest that infection structures are constantly adjusting in order to overcome host barriers (Hajek & St. Leger, 1994).

After penetration, yeast-like blastospores colonize the hemocoel and the host may be killed by mechanical damage produced by fungal growth or by the production of toxins (Butt & Goettel, 2000). However, to succeed and complete their cycle, entomopathogenic fungi depend on abiotic factors such as humidity, temperature, pH (Pell *et al*., 2010) and the ability to utilize available nutrients on the cuticle surface. While the adhesion of conidia depends on hydrophobic substances, the blastospore depends on hydrophilic substances (Holder & Keyhani, 2005), demonstrating that there is a difference between the needs in each infecting form. After host death, the fungus passes through the epidermis, procuticle and finally, emerges from the epicuticle, leaving the host body. Then,

reproductive structures are formed and conidiogenesis occurs. Conidia so formed will need a new host to start this cycle or become quiescent in the soil. Thus, these fungi can be considered hemibiotrophic fungi, presenting a parasitic phase, which is when it develops inside its host and saprophytic phases when its host dies (Shah & Pell, 2003).

Many attributes determine the virulence of entomopathogenic fungi to their hosts, with the production of degradative enzymes being of extreme importance (Tiago *et al*., 2002). This virulence determines how the host is affected by the pathogen; host-specific toxins can be determinants of pathogenicity and virulence in plants (Dickman, 2007) or animals. It is likely that the virulence of the fungi vary according to host species and geographic region considered (Silva, 2001). Since the success of an entomopathogenic fungus in biological control depends largely on the choice of the fungal isolate, it is necessary to screen an array of fungal strains to for the capacity to choose the best candidates to be use in mosquito control (Mnyone *et al*., 2009).

1.4 Screening fungal isolates

Fungal isolates are commonly obtained by isolation from soil samples, using selective media or with the insect bait method (Meyling, 2007). The bait method works by placing bait (in this case, a live insect), on collected soil and leaving it for a few days to become infected and die. The dead insect is then surface-sterilized and put in a moist chamber until fungus sporulation. These fungi can be isolated and subsequently screened, considering characteristics of interest. For example, inoculating the fungus in live insects or others arthropods, recording daily their survival and selecting the isolate that killed them more quickly (Vu *et al*., 2007; Herlinda *et al*., 2008; Barci *et al*., 2009) or, that produce certainly substances (Guimarães *et al*., 2006) or, as in our study, isolates able to infect two mosquitoes at the same time.

Screening of fungi for use against mosquitoes is usually based on assays of mortality of infected mosquitoes. However, a disadvantage of this method is that the adults may survive for up to 20 days. Additionally, this set-up will select the most virulent isolates, discarding those that would take longer to kill but could cause useful sublethal effects.

Many studies have been undertaken to observe the development of fungi on cuticles of insects and other arthropods, and on cuticle compounds, for example in scarab larvae (Yaginuma *et al*., 2006) on locust wings (Wang & St. Leger, 2005), with fleas (Melo *et al.,* 2007) and with ticks (Bittencourt *et al.*, 1999, Garcia *et al*., 2004; Arruda *et al*., 2005). These studies have shown that pathogen responses can vary with the cuticles as reported by Wang & St. Leger (2005). These authors studied the development of a *Metarhizium* isolate on the host and non-host cuticle and with some cuticular compounds.

Thus, we propose here a different approach to fungal isolate screening. We investigated the development of infection structures of Brazilian isolates of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* on the cuticles of wings of the mosquitoes *Ae. aegypti* and *An. aquasalis*, based on the formation of infective structures (appressorium) on the wing cuticles. It is intended that this technique will facilitate isolate screening by reducing the time spent in screening and also allow the inclusion of less virulent isolates.

2. OBJECTIVE

Develop a technique of isolate screening based on the formation of appressoria by isolates of *B. bassiana* and *M. anisopliae* that can infect both mosquito vectors *Ae. aegypti* and *An. aquasalis*.

3. MATERIALS AND METHODS

3.1 Culicids

We worked with the *Aedes aegypti* strain PPCampos, originally from Campos dos Goytacazes, RJ, Brazil. These were reared in cages at 27 ± 1°C, 75 ± 5% RH and 12L:12D photoperiod. Oviposition occurred in beakers half-filled with water and lined with filter paper. Egg eclosion was stimulated by total immersion of the filter paper in water. Larvae were kept in plastic trays and fed with turtle food. Pupae were separated and placed in adult emergence cages. Adults were fed with glucose solution offered *ad libitum* in cotton and weekly mouse blood meals for females. *Anopheles aquasalis* were obtained from the rearing of CPqRR-FIOCRUZ (René Rachou Research Center).

3.2 Fungi

The majority of fungal isolates used (72) were isolated from soil samples using beetle larvae (*Tenebrio molitor*) as live bait (C. Moreira – unpublished data). Nine isolates were obtained from UFRPE (Federal Rural University of Pernambuco) and two from the company Itaforte BioProdutos. The latter isolates are the basis for the biopesticide products Boveril® and Metarril® (Table 1).

Table 1. Sources of isolates of entomopathogenic fungi used for screening.

Fungi	Isolate	Host/Source	Geographical Origin
Beauveria bassiana	C76B, J57A, J80B, L2A L8A, L46C, L56A, L72A S6C, S33B, S52C, S55D S71B, S71D, S79A, S79C	Soil using live bait *Tenebrio molitor L. (Coleoptera: Tenebrionidae)*	Araponga-MG, Brazil
	Boveril®	Itaforte	Brazil
	URPE-3	*Rhynchophorus palmarum L. (Coleoptera: Curculionidae)*	Cabo-PE, Brazil
	URPE-4	*Membracis sp. L. (Hemiptera: Membracidae)*	Recife-PE, Brazil
	URPE-14	Soil	Escada-PE, Brazil
	URPE-18	Soil	Cabo-PE, Brazil
	URPE-22	Bug	Chapada Diamantina-BA, Brazil
Metarhizium anisopliae	C26A, C46A, C54B, C55A C66A, C84A, C86C,J2B J8C, J11B, J15D, J18A J21C, J25A, J27A, J30B J32A, J33C, J38A, J42A J43A, J46A, J52B, J54B J60A, J65C, J75A, J75B J81C, L10B, L13A, L13D L54A, L58A, L58D, L60A L60B, L62B, S2A, S3A S10A, S11A, S12A, S13B S13C, S24C, S31B, S33C S34B, S35B, S51A, S61A S62B, S67A, S68A, S77B	Soil using live bait *Tenebrio molitor L. (Coleoptera: Tenebrionidae)*	Araponga-MG, Brazil
	Metarril®	Itaforte	Brazil
	URPE-11	*Mahanarva posticata (Stål, 1855) (Hemiptera: Cercopidae)*	Recife-PE, Brazil
	URPE-19	Soil	Vitória-PE, Brazil
	URPE-30	Soil	Moreno-PE, Brazil
	URPE-32	Soil	Itambé-PE, Brazil

Eighty-three fungal isolates were grown on Potato Dextrose Agar (PDA) at 27°C, for the first step of screening (*Ae. aegypti* wings). Conidial suspensions were prepared from fungal material removed from the plates, diluted in sterile distilled water plus Tween 80® and vortex-mixed. The standard suspension was filtered through sterile gauze to remove mycelial mat and dilutions were prepared and quantified by counting in a Neubauer hemocytometer, to make up final suspensions of $1x10^5$ conidia ml^{-1}.

Conidial viability (percentage of germinated conidia) was determined 24h after use by placing spore suspensions on Petri dishes with water agar. These were sealed and incubated at 27°C for 24 h. After this, they were examined at 40x magnification. For each isolate, approximately 200 conidia were examined.

3.3 Improving the screening technique

The technique described by Wang & St. Leger (2005) to study formation of appressoria on insect cuticles begins by killing the insects by freezing and dissecting their wings. These are surface-sterilized in 5% sodium hypochlorite for 5 min and are then rinsed 4 times (5 min each) in sterile distilled water. Then, the wings are inoculated with the conidial suspension and placed in Petri dishes with water agar. Based on the idea that to have infection, there must be recognition of the host cuticle, we conducted preliminary tests with isolates obtained from soil, using *T. molitor* as live bait. For this, we used Wang and St. Leger's (2005) methodology to observe the development of some isolates on the membranous wings of these insects. This strategy would be to familiarize ourselves with the structures formed and make minor adjustments to then test the technique in mosquitoes. However, fungal isolates tested did not germinate in *T. molitor* wings.

We adjusted the technique described by Wang and St. Leger (2005). After the insects are killed and had their wings dissected, we added a step: we rinsed the wings in alcohol 70%, to break the surface tension. The next step remained the same; leaving them in 5% sodium hypochlorite for 5 min. Instead of leaving 5 min in each change of water, we just rinsed the wings in sterile distilled water, reducing the time in the bath water. Then, we inoculated fungi

suspension on *Tenebrio* wings. These were placed on water-agar Petri dish, amounting to 5 wings per plate and incubated at 27°C. We made slides, with lactophenol, lacto-fuchsin stain and lactophenol cotton blue stains, in order to select which stain facilitated the observation of the isolates of *B. bassiana* and *M. anisopliae*.

These slides was observed in light microscope at 40x and 100x magnification, 24h, 30h and 48h post-infection in order to determine the best time to make the observations. Then, we verified the development of the isolates on *Tenebrio* wings, selecting the steps that were most satisfactory.

3.4 Germination and appressorium formation on *Aedes* wings

With the adjustments described above, we carried out tests with adult mosquitoes of mixed sexes and ages. We inoculated 83 fungal suspensions (22 isolates of *B. bassiana* and 61 isolates of *M. anisopliae*) on wings of *Ae. aegypti*. Each wing was inoculated with 0,5 µl of fungal suspension and for each isolate, we used one plate containing 5 wings. As a positive control, we used *T. molitor* wings inoculated with *M. anisopliae* isolate J27A.

A period after the inoculation, we removed these wings from the Petri dishes and mounted them on slides with stains. We observed and counted the conidia, germinated conidia and appressoria at 40x and 100x magnifications. We recorded the percentage of conidia that germinated and that showed the differentiation of the germ tube into appressoria. We considered appressorium formation when there was a thickening of the hyphae (Figure 1-3).

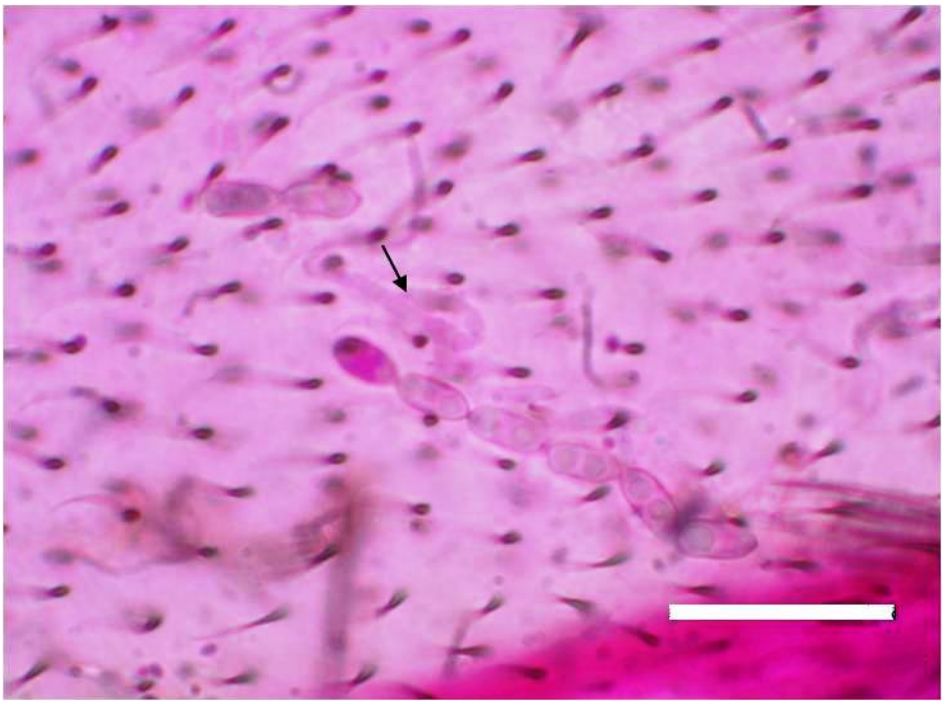

Figure 1. Conidia of germinated *Metarhizium anisopliae* on the wing cuticle of *Aedes aegypti* mosquito. The arrow shows the germ tube (Bar = 10 µm).

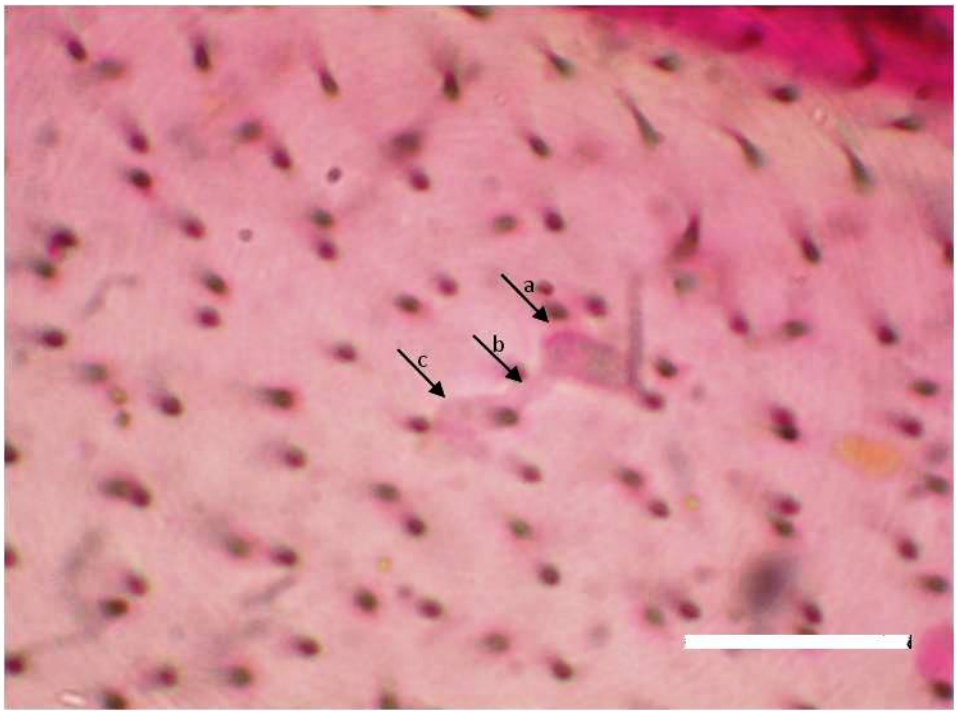

Figure 2. Germinated conidium of *Metarhizium anisopliae* and its appressorium on the wing cuticle of *Aedes aegypti*. Arrows **a** – conidium; **b** – germ-tube; **c** – hyphal differentiation into appressorium (Bar = 10 µm).

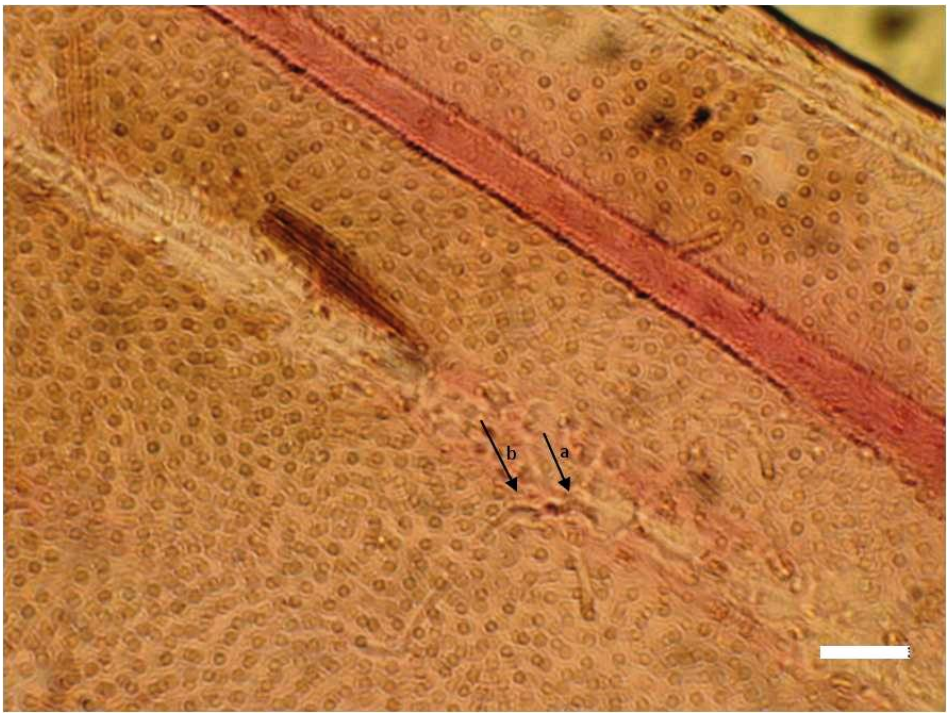

Figure 3. Germinated conidium of *Beauveria bassiana* and its appressorium on the wing cuticle of *Aedes aegypti*. Arrows **a** – conidium; **b** – hyphal differentiation into appressorium (Bar = 10 µm).

3.5 Germination and appressorium formation on *Anopheles* wings

For screening against *An. aquasalis*, we selected the isolates that had germinated and formed appressoria on *Ae. aegypti.* For logistical reasons we were restricted to 40 of these isolates. The procedure was as described above.

3.6 Test with live *Aedes aegypti*

For a pilot assay of virulence, we selected 8 fungal isolates that showed good germination and appressorium formation on wings of both mosquito species (*M. anisopliae* J43A, *M. anisopliae* J75A*, M. anisopliae* Metarril®*, M. anisopliae* URPE 11, *M. anisopliae* URPE 32, *B. bassiana* URPE 4, *B. bassiana* URPE 14 and *B. bassiana* Boveril®). We used a further isolate, *M. anisopliae* J33C that did not germinate on *Ae. aegypti* wings, so could be used to check whether an isolate that did not germinate on the wing would not infect the live mosquitoes, and we used a water plus Tween control.

For each treatment, we separated 10 adult females of 1-8 days age, fed with glucose solution *ad libitum* in cotton. These placed in gauze-covered cages

(22 cm in diameter x 19 cm). Pieces of absorbent paper were soaked in 5ml of fungal suspensions of 1 x 10^7 µl ml^{-1} and placed in the cages, where they remained for 24 h, simulating the way the product could be used in the field. After this time, the inoculum was removed from the cage and we observed survival for 15 days, separating the dead mosquitoes daily in moisture chambers to determine sporulation.

This experiment was done in randomized blocks with 3 repetitions and mosquito survival data were analyzed using the program Statistica 7.0. and Kaplan-Meier pairwise method was used.

4. RESULTS

4.1 Fungi

Conidial viability was variable but where isolates were used against both mosquito species, was generally consistent between the two assays (Table 2).

Table 2. Percentage conidial viability of the 83 fungal isolates used on wings of *Aedes aegypti* and 40 of these on wings of *Anopheles aquasalis*.

Fungi	Isolate	Viability on wings of *Aedes aegypti* (%)	Viability on wings of *Anopheles aquasalis* (%)
Beauveria bassiana	C76B	90	
	J57A	74	
	J80B	80	79
	L2A	86	
	L8A	93	
	L46C	82	79
	L56A	84	
	L72A	90	
	S6C	72	70
	S33B	82	
	S52C	62	61
	S55D	89	
	S71B	80	
	S71D	85	
	S79A	52	
	S79C	96	
	Boveril®	81	82
	URPE-3	99	
	URPE-4	92	90
	URPE-14	74	75
	URPE-18	79	
	URPE-22	95	
Metarhizium anisopliae	C26A	28	28
	C46A	93	78
	C54B	68	
	C55A	2	2
	C66A	95	95
	C84A	25	24
	C86C	10	2
	J2B	72	72
	J8C	85	
	J11B	93	19
	J15D	19	37
	J18A	88	45
	J21C	45	7
	J25A	47	30
	J27A	15	76
	J30B	98	81
	J32A	81	93
	J33C	85	
	J38A	5	5
	J42A	96	18
	J43A	80	73
	J46A	38	1
	J52B	93	90

Continue…

Table 2. Continue…

Fungi	Isolate	Viability on wings of *Aedes aegypti* (%)	Viability on wings of *Anopheles aquasalis* (%)
Metarhizium anisopliae	J54B	85	85
	J60A	49	46
	J65C	85	41
	J75A	83	81
	J75B	88	72
	J81C	94	42
	L10B	49	
	L13A	5	
	L13D	63	
	L54A	43	41
	L58A	72	
	L58D	22	
	L60A	33	30
	L60B	5	
	L62B	10	
	S2A	13	
	S3A	59	
	S10A	52	
	S11A	11	
	S12A	88	
	S13B	77	
	S13C	80	
	S24C	96	
	S31B	24	
	S33C	93	
	S34B	94	
	S35B	86	
	S51A	21	
	S61A	12	
	S62B	91	
	S67A	61	
	S68A	74	
	S77B	86	
	Metarril®	62	60
	URPE-11	99	99
	URPE-19	92	91
	URPE-30	98	99
	URPE-32	96	97

4.2 Improving the screening technique

The stain with more satisfactory results for observation of the fungus on the mosquito wing slides was lacto-fuchsin, although it also stained the wings (Figure 4).

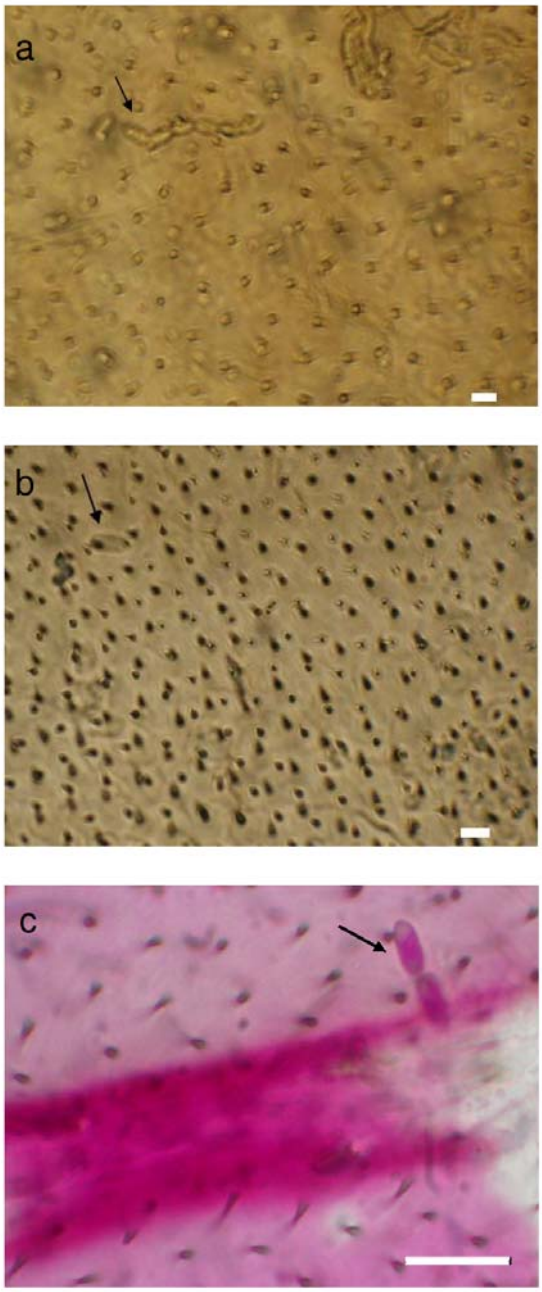

Figure 4. Selection of stains for observation of germinating conidia of *Metarhizium anisopliae*. (a) shows a slide made with lactophenol, (b) with lactophenol cotton blue, (c) lacto-fuchsin. The arrows indicate fungal conidia (Bar = 10 µm).

In pilot experiments inoculating fungi in *Tenebrio* wing cuticles, we determined 24 h post-inoculation was too soon to observe germination or appressorium formation, compared with 30 and 48 h. However, at 48 h, the hyphae had grown across the wing, making it difficult to observe conidia and verify appressorium formation. Saprophytes also began to contaminate the material at this point. Therefore, we decided to use 30 h post-inoculation to make the slides.

Of the fungi tested on *Tenebrio* wings, isolate J27A presented the highest amount of apressorium formation so was subsequently used as a positive control.

4.3 Germination and appressorium formation on *Aedes* wings

Of 83 isolates tested in *Ae. aegypti* between 3 did not germinate, 13 germinated but did not form appressoria while 67 germinated and formed appressoria (Table 4), with a range of values for both variables.

4.4 Germination and appressorium formation on *Anopheles* wings

All of the 40 isolates tested in *An. aquasalis* germinated although 8 of these did not form appressoria (Table 3). There was no apparent correlation between the values of these two variables. Note that here, isolate J43A on *Aedes* wings was used as the positive control.

Table 3. Percentage of the isolates of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* that germinate (G%) and formed appressorium (A%) on wing cuticles of *Aedes aegypti* and *Anopheles aquasalis* (G% is the percentage of number of germinated conidia/total of conidia; A% is the percentage of number of conidia that formed appressorium/number of germinated conidia).

Fungi	Isolate	*Aedes aegypti*		*Anopheles aquasalis*	
		G%	A%	G%	A%
Metarhizium anisopliae	C76B	55,56	40,00		
	J57A	14,29	0,00		
	J80B	73,68	7,14	100,00	6,25
	L2A	97,50	0,00		
	L8A	20,83	0,00		
	L46C	30,43	14,29	70,00	0,00
	L56A	80,00	16,67		
	L72A	0,00	0,00		
	S6C	87,88	41,38	33,33	0,00
	S33B	50,00	16,67		
	S52C	86,21	8,00	100,00	10,42
	S55D	86,11	22,58		
	S71B	71,43	40,00		
	S71D	2,27	0,00		

Continue…

Table 3. Continue…

Fungi	Isolate	*Aedes aegypti*		*Anopheles aquasalis*	
		G%	A%	G%	A%
Beauveria bassiana	S79A	62,07	11,11		
	S79C	87,50	14,29		
	Boveril®	29,17	28,57	78,05	43,75
	URPE-3	86,49	40,63		
	URPE-4	77,78	64,29	77,78	14,29
	URPE-14	28,95	9,09	91,89	8,82
	URPE-18	61,70	44,83		
	URPE-22	61,29	15,79		
Metarhizium anisopliae	C26A	78,95	20,00	75,00	20,83
	C46A	89,29	28,00	100,00	25,58
	C54B	8,77	0,00		
	C55A	50,00	11,11	4,35	0,00
	C66A	80,00	33,33	80,00	45,45
	C84A	100,00	30,00	93,33	14,29
	C86C	67,50	3,70	5,06	0,00
	J2B	90,48	31,58	93,64	23,30
	J8C	52,63	0,00		
	J11B	96,43	20,99	89,90	14,61
	J15D	60,00	33,33	84,21	31,25
	J18A	80,00	25,00	87,10	37,04
	J21C	100,00	38,46	89,66	23,08
	J25A	90,91	25,00	29,41	0,00
	J27A	88,89	6,25	100,00	25,00
	J30B	93,48	18,60	90,91	35,00
	J32A	84,04	49,37	100,00	23,08
	J33C	0,00	0,00		
	J38A	45,45	10,00	4,55	0,00
	J42A	91,67	9,09	63,64	28,57
	J43A	94,44	11,76	94,59	17,14
	J46A	75,00	22,22	11,11	0,00
	J52B	75,68	42,86	66,67	37,50
	J54B	100,00	16,67	91,67	36,36
	J60A	81,25	61,54	96,61	33,33
	J65C	94,44	41,18	38,52	26,92
	J75A	84,38	62,96	42,65	3,45
	J75B	93,62	20,45	75,36	21,15
	J81C	60,00	33,33	83,13	24,64
	L10B	68,09	21,88		
	L13A	20,00	0,00		
	L13D	80,43	43,24		

Continue…

Table 3. Continue…

Fungi	Isolate	*Aedes aegypti*		*Anopheles aquasalis*	
		G%	A%	G%	A%
Metarhizium anisopliae	L54A	72,22	46,15	78,57	13,64
	L58A	81,82	55,56		
	L58D	76,47	0,00		
	L60A	84,21	56,25	91,84	60,00
	L60B	48,39	0,00		
	L62B	82,61	5,26		
	S2A	0,00	0,00		
	S3A	37,84	10,71		
	S10A	72,73	0,00		
	S11A	42,86	0,00		
	S12A	60,00	0,00		
	S13B	97,40	50,67		
	S13C	66,67	0,00		
	S24C	75,68	7,14		
	S31B	41,59	31,46		
	S33C	86,21	70,00		
	S34B	54,55	8,33		
	S35B	8,70	50,00		
	S51A	47,06	37,50		
	S61A	11,86	28,57		
	S62B	46,67	28,57		
	S67A	50,00	11,11		
	S68A	24,00	27,78		
	S77B	82,35	28,57		
	Metarril®	31,25	80,00	85,71	16,67
	URPE-11	100,00	33,33	91,89	50,00
	URPE-19	37,78	23,53	100,00	47,22
	URPE-30	86,67	11,54	93,02	0,00
	URPE-32	92,31	54,17	87,50	28,57

4.5 Test with live *Aedes aegypti*

The pilot bioassay showed promising initial results in that there was generally a higher mortality in the fungus-treated insects than the control (Table 4). Between days 5 and 15, however, there were very few deaths, implying that there may have been a problem in establishing infection in this setup.

Table 4. Mortality of *Aedes aegypti* mosquitoes per day of total 10 adults females 1-8 days old, during 15 days, randomized in three blocks.

Block	Fungi Isolates	Total number of mosquitoes	Number of dead mosquito per day															Final number of mosquitoes
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Block 1	Water plus Tween®	10																10
	J33C	10																10
	J43A	10			1													9
	J75A	10	1															9
	Metarril®	10																10
	URPE 11	10																10
	URPE 32	10	1															9
	URPE 4	10																10
	URPE 14	10				1												9
	Boveril®	10				1				1								8
Block 2	Water plus Tween®	10		1														9
	J33C	10																10
	J43A	10	1			1												8
	J75A	10	1			1		1										7
	Metarril®	10				1												9
	URPE 11	10	1			2												7
	URPE 32	10				2												8
	URPE 4	10		1		2												7
	URPE 14	10			1	1												8
	Boveril®	10																10

Continue...

Table 4. Continue…

Block	Fungi Isolates	Total number of mosquitoes	Number of dead mosquito per day															Final number of mosquitoes
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Block 3	Water plus Tween®	10														1		9
	J33C	10																10
	J43A	10	1		1													8
	J75A	10																10
	Metarril®	10																10
	URPE 11	10	1															9
	URPE 32	10		1	1													8
	URPE 4	10	1				1											8
	URPE 14	10																10
	Boveril®	10			1	1												8

5. DISCUSSION

Due to problems with synthetic insecticides (Lacey & Undeen, 1986; Hemingway & Ranson, 2000; Lacey *et al.,* 2001; Shiff, 2002; Hargreaves *et al.*, 2003), biological control of mosquitoes with entomopathogenic fungi is an expanding area. Fungi infect all stages of mosquito development, having shown satisfactory results against eggs (Luz *et al*., 2008), larvae (Mohanty *et al.*, 2008) and adults (Mnyone *et al*, 2009; Leles *et al.*, 2010). Meanwhile, biological control of larvae using bacterial pathogens is considered a cheap, easy and environmentally friendly method (Kannan *et al*., 2008), but problems that can occur are the dissipation of the control agent in the aquatic environment and killing of non-target organisms. Thus, more efforts have been done to develop agents against adult mosquitoes (Kanzok & Jacobs-Lorena, 2006; Kannan *et al*., 2008).

Several studies have demonstrated the potential of entomopathogenic fungi, both in the control of vector populations and in acting on the vectorial capacity of mosquitoes (Blanford *et al.* 2005; Scholte *et al.* 2004; 2005; 2006; 2007; Thomas & Read, 2007; de Paula *et al*., 2008; Kannan *et al*., 2008; Mohanty *et al.* 2008; Leles *et al.*, 2010; Mnyone *et al*., 2011; Howard *et al*., 2010a). Reducing mosquito lifespan interferes with blood feeding, thus affecting the pathogen acquisition and transmission (Scholte *et al*., 2007; Mohanty *et al*., 2008) and this is a key factor in the use of entomopathogenic fungi.

The traditional way to screen entomopathogenic fungi is to inoculate the fungus in live organisms. In our study, we focused the technique that allows the screening of some isolates based on the formation of infection structures called appressoria, using the wing cuticle. Although we attempted to obtain isolates from soil using larvae of *Musca domestica* (Diptera) as live bait (data not shown), this was not successful, probably due to rapid pupation of the larvae prior to death from fungal infection.

For the assays of germination and appressorium formation on mosquito wings, we found lactophenol alone to be inadequate for visualization of the fungal structures. Although lactophenol cotton blue did stain the fungal structures, it was not as clear as when we used lacto-fuchsin stain, especially for conidia of *M. anisopliae*.

On the slides, we could see some long hyphae growing close to each other, forming aggregations. We also noted some subterminal appressoria, as described by Wang & St. Leger (2005). It could be that the two phenomena are connected, perhaps resulting from unsuccessful attempts to invade the host or as a means of obtaining nutrients to continue growth. Although cuticle topography appears to influence the growth form of *M. anisopliae* (St. Leger *et al*., 1990a), our study was inconclusive in this respect. In line with Arruda *et al*. (2005), then, it is likely that other factors may have influenced fungal development such as chemical clues linked to cuticle compounds.

According to Moino *et al*. (2002) the thickening of hyphae may be due to the translocation of the conidial cytoplasmatic content to facilitate the enzymatic synthesis necessary for the penetration phase. However, *B. bassiana* in general had low percentages of appressorium formation. Some studies have shown that the germ-tube can penetrate the host cuticle without appressorium formation (Melo *et al*., 2007), as with plants where penetration occurred through a small hole (Quesada-Moraga *et al*., 2006); this may suggest that isolates that did not form appressoria could still be of interest.

Our screening technique was faster than the conventional method. Screening of isolates for mosquito control usually begins with assays of daily survival of infected mosquitoes, in order to select isolates that kill quickly. One disadvantage of this method is that the adults survive up to 20 days so the experiment is prolonged as some fungi take several days to kill them. It also leads to a selection of the most virulent isolates, discarding those that would take longer to kill but might cause significant sublethal effects for the control of mosquitoes and their vectorial capacity. Similarities in the two methods are to be found in the need of adult mosquitoes and sporulating fungi. As mosquitoes and fungi take the same time to develop in both set-ups, the time spent with this is equal, as is the preparation of spore suspensions.

Thus, what really varies between the methods is the next step, between inoculation and the final result. In the traditional method, we have to wait for infected mosquitoes to die, while using our technique of making the observation of infection in the cuticle structures of the wings, we can use the newly emerged mosquitoes, and our results will be obtained in just 30 hours, when there is the recognition and then, infection structure formation. This implies a faster and

more effective screening (as many isolates that were discarded in the other method are kept here) of fungal isolates able to infect these insects.

Furthermore, our results support the notion that the interaction between host and pathogen can be influenced by differences in the cuticular components on mosquito wings, since there were differences in the development of the fungi on mosquito cuticle, in line with results of Wang & St. Leger (2005). Fungi need an external resource of nutrients to germinate (Pedrini *et al*., 2007) and the medium interferes in their germination, viability and virulence (Rangel *et al*., 2008). Different expression of genes encoding cuticle-degrading enzymes, cell wall proteins, toxins and toxin-producing enzymes have been observed on different insect cuticles (Freimoser *et al*., 2005). Meanwhile, germination on non-host cuticle is limited (Wang & St. Leger, 2005; Jarrold *et al.,* 2007), this perhaps being one of the factors that influenced the development of our isolates on mosquito cuticles. Wang and St. Leger (2005) suggested that there could be localized formation of appressoria on non-host cuticle. In line with this, some of our isolates formed small numbers of appressoria in our study.

Analyzing the studies that deal with infection progression, we have a pattern of development of these fungi in the host cuticle, demonstrating that the time of infection process varies among fungi (Castrillo *et al*., 2005). Usually, the sequence of events is: conidia attachment; conidia germination and development of a germ-tube that colonizes the host cuticle, 24-48 h post-infection; germ-tube penetration or development of appressorium; 24-48 h post-infection (Arruda *et al*., 2005). There is variation in the timing of these events: thus, in one study, for *M. anisopliae* on locust wings, 73% germination was achieved at 24 h, with some appressorium formation, while on cicada wings there was 54% germination of *M. anisopliae* at 24h and appressoria were rarely observed. Meanwhile, in beetles, this value was just 4% with no appressoria. (Wang & St. Leger, 2005). In fleas, adherence occurred 2 h after inoculation, there was germ-tube germination 26 h post-inoculation and there was also thickening and branching of hyphae, with no appressoria.

Observing these temporal differences, we can confirm that 30 h post-infection was satisfactory to our study of screening fugal isolates based on appressoria formation, because after this time, saprophytic structures were

formed, suggesting that there was a dearth of nutrients available on the wing cuticle.

The mosquito must come in contact with the conidia of the fungus and the conidia have to adhere to its surface to be established infection (Charnley, 1994). Thus, the success of infection is determined by host contact with treated surface and concentration of infective conidia (Vandenberg *et al.*, 1998). Therefore, our survival bioassay suggests that there may have been an insufficient concentration of fungal conidia or insufficient time for the assay, unfavorable environmental conditions to fungi development or there may have been a repulsion of mosquitoes in relation to the paper treated with the fungus. Scholte *et al.* (2003) reported a moderate repellence of *An. gambiae* for dry conidia of *M. anisopliae*. However, when the suspension is formulated with oil, this effect disappears (Mnyone *et al.,* 2010). The relative high mortality in the first four days suggests that mortality may have been caused by the change of environment for testing.

The tests with *Anopheles* cuticles and with live mosquitoes are not yet concluded. The *Anopheles* tests should continue when more adult insects are available to obtain more wings, since just 40 of the 67 isolates that were pre-selected in *Aedes* were tested. The assay in live mosquitoes needs refining and repeating; of the 67 isolates able to form structures in infective *Aedes*, only 8 were tested (of 10 treatments, one control and one was an isolate that was not formed appressoria). Our intention is to conduct the test with live mosquitoes of both species and with all isolates that formed infective structure on the wing cuticles, to confirm that our technique is really effective and determine if the mortality test is indeed a less effective means of screening.

Among the isolates tested, 5 of *B. bassiana* and 27 of *M. anisopliae* formed appressoria in both mosquitoes, indicating promise for their use in control. The commercial products Boveril® and Metarril® show potential which is of great interest as these are registered products with a market history, besides presenting a formulation already developed and field tested appropriate to conditions in Brazil.

Further studies are necessary to screen fungi as possible candidates. Following this, it will be necessary to obtain an understanding of the ecology of

the fungus in field tests, and studying the formulation and application for the control of mosquitoes of different genera.

6. CONCLUSION

Improving the screening fungal technique based on the formation of appressoria using mosquito wings, allowed us to streamline the selection of potential fungi for control of insect vectors. Furthermore, this study allowed the observation of fungi development in the mosquitoes cuticle and demonstrated that these phases vary with fungal isolate and host cuticle, and are very important in selecting isolates for biological control of insects. Some isolates of *B. bassiana* and *M. anisopliae* had infection structures (appressoria) in both mosquitoes proving to be potential agents for biological control.

APPENDICES

Number of conidia (C), number of germinated conidia (G) and number of appressoria formatted (A) of *Beauveria bassiana* isolates in each one of five wings of *Aedes aegypti*.

Beauveria bassiana	***Aedes aegypti***														
	1º Wing			**2º Wing**			**3º Wing**			**4º Wing**			**5º Wing**		
Isolate	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**
C76B	2	0	0	1	1	0	3	2	2	1	1	0	2	1	0
J57A	2	1	0	2	0	0	1	0	0	2	0	0	0	0	0
J80B	1	0	0	7	7	1	2	0	0	3	2	0	6	5	0
L2A	8	7	0	17	17	0	2	2	0	6	6	0	7	7	0
L8A	7	4	0	5	1	0	2	0	0	5	0	0	5	0	0
L46C	4	0	0	3	0	0	7	3	0	3	1	0	6	3	1
L56A	4	3	0	12	12	4	2	1	0	6	3	0	6	5	0
L72A	20	0	0	12	0	0	5	0	0	7	0	0	4	0	0
S6C	7	6	0	4	3	0	4	3	2	6	5	3	12	12	7
S33B	6	2	0	7	5	0	6	2	0	3	2	2	2	1	0
S52C	4	4	0	5	5	0	11	11	2	4	0	0	5	5	0
S55D	10	6	2	22	19	7	35	35	5	2	2	0	3	0	0
S71B	3	2	0	2	2	1	3	1	1	5	5	2	1	0	0
S71D	10	0	0	8	1	0	7	0	0	17	0	0	2	0	0
S79A	10	9	1	4	2	0	6	3	0	6	4	1	3	0	0
S79C	1	1	0	1	0	0	7	6	0	4	4	1	3	3	1
Boveril®	5	3	1	7	3	0	4	0	0	4	0	0	4	1	1
URPE-3	12	9	2	10	8	5	8	8	2	5	5	2	2	2	2
URPE-4	1	1	0	14	11	9	1	1	0	1	0	0	1	1	0
URPE-14	10	0	0	13	5	0	7	2	0	5	2	1	3	2	0
URPE-18	18	13	8	5	3	0	6	3	2	8	5	1	10	5	2
URPE-22	4	0	0	3	1	0	6	4	0	18	14	3	0	0	0

Number of conidia (C), number of germinated conidia (G) and number of appressoria formatted (A) of *Metarhizium anisopliae* isolates in each one of five wings of *Aedes aegypti*.

Metarhizium anisopliae	***Aedes aegypti***														
	1º Wing			**2º Wing**			**3º Wing**			**4º Wing**			**5º Wing**		
Isolate	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**
C26A	9	6	0	7	7	0	19	14	6	3	3	0	0	0	0
C46A	4	4	2	7	6	0	5	4	2	8	8	2	4	3	1
C54B	22	3	0	33	4	0	38	3	0	2	0	0	19	0	0
C55A	6	2	0	0	0	0	3	2	1	3	3	0	6	2	0
C66A	16	11	2	17	13	7	8	8	3	2	2	0	2	2	0
C84A	7	7	2	3	3	0	7	7	1	0	0	0	3	3	3
C86C	5	5	0	13	8	0	5	5	1	10	5	0	7	4	0
J2B	6	6	4	8	8	2	2	0	0	0	0	0	5	5	0
J8C	4	1	0	4	2	0	9	7	0	1	0	0	1	0	0
J11B	11	8	2	26	26	5	2	2	0	15	15	5	30	30	5
J15D	6	6	2	3	3	1	2	0	0	4	0	0	0	0	0
J18A	1	0	0	4	0	0	2	2	2	17	17	3	1	1	0
J21C	33	33	16	8	8	2	1	1	0	10	10	2	0	0	0
J25A	4	3	0	4	4	0	13	12	4	0	0	0	1	1	1
J27A	7	7	0	3	3	0	2	2	0	2	2	0	4	2	0
J30B	11	9	0	5	4	0	14	14	6	12	12	0	4	4	2
J32A	12	7	0	14	7	3	30	28	18	16	16	5	22	21	13
J33C	17	0	0	18	0	0	4	0	0	10	0	0	4	0	0
J38A	1	1	0	13	7	0	10	6	1	6	2	1	14	4	0
J42A	3	3	0	3	3	0	1	1	0	1	0	0	4	4	1

Continue…

Continue…

Metarhizium anisopliae	*Aedes aegypti*														
	1º Wing			2º Wing			3º Wing			4º Wing			5º Wing		
Isolate	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**
J43A	2	2	0	3	2	1	4	4	1	7	7	0	2	2	0
J46A	4	2	1	1	1	0	1	0	0	2	2	1	4	4	0
J52B	6	4	0	14	12	7	8	4	2	9	8	3	0	0	0
J54B	1	1	0	6	6	0	7	7	3	3	3	0	1	1	0
J60A	8	7	5	1	1	0	3	1	0	1	1	1	3	3	2
J65C	11	10	2	9	9	7	9	9	3	6	5	2	1	1	0
J75A	6	2	1	5	5	5	2	2	0	18	18	11	1	0	0
J75B	22	19	3	6	6	2	6	6	2	2	2	0	11	11	2
J81C	3	1	0	1	1	1	3	3	0	2	1	1	1	0	0
L10B	3	3	2	36	21	3	4	4	1	2	2	0	2	2	1
L13A	1	0	0	1	0	0	2	1	0	1	0	0	0	0	0
L13D	4	4	2	12	11	3	10	8	5	13	8	3	7	6	3
L54A	4	3	1	3	0	0	2	2	0	0	0	0	9	8	5
L58A	3	3	0	10	10	6	20	16	12	8	4	1	3	3	1
L60A	6	5	2	13	12	8	10	8	3	7	6	5	2	1	0
L60B	14	8	0	2	2	0	10	3	0	1	1	0	4	1	0
L62B	2	0	0	13	13	0	5	5	1	1	0	0	2	1	0
S2A	12	0	0	4	0	0	7	0	0	8	0	0	18	0	0
S3A	8	4	3	16	0	0	32	18	0	2	0	0	16	6	0
S10A	1	0	0	2	1	0	1	1	0	1	1	0	6	5	0
S11A	3	2	0	1	1	0	3	0	0	0	0	0	0	0	0
S12A	3	1	0	3	0	0	20	16	0	2	0	0	2	1	0
S13B	28	26	8	18	18	8	4	4	2	14	14	8	13	13	12
S13C	2	2	0	2	2	0	1	0	0	2	0	0	2	2	0
S24C	1	1	0	7	6	0	1	1	0	25	19	2	3	1	0
S31B	43	27	10	41	10	3	49	22	10	33	18	5	48	12	0
S33C	15	12	7	5	5	4	18	13	10	10	10	7	10	10	7
S34B	4	0	0	19	13	1	11	5	1	6	3	0	4	3	0
S35B	16	0	0	3	1	1	1	0	0	1	1	0	2	0	0
S51A	1	1	0	1	0	0	1	0	0	11	5	3	3	2	0
S61A	2	0	0	7	5	2	37	2	0	9	0	0	4	0	0
S62B	14	3	2	3	3	0	4	2	2	4	4	0	5	2	0
S67A	9	2	0	1	1	0	1	0	0	6	6	1	1	0	0
S68A	60	7	3	4	3	1	2	0	0	2	1	0	7	7	1
S77B	6	5	1	4	4	0	14	11	2	4	3	2	6	5	3
Metarril®	2	0	0	11	2	2	0	0	0	1	1	0	2	2	2
URPE-11	21	21	5	58	58	26	1	1	0	45	45	12	19	19	5
URPE-19	15	7	3	8	5	1	13	0	0	6	3	0	3	2	0
URPE-30	12	10	0	11	11	3	4	3	0	1	0	0	2	2	0
URPE-32	5	5	2	3	3	3	5	5	2	5	4	4	8	7	2

Number of conidia (C), number of germinated conidia (G) and number of appressoria formed (A) of *Beauveria bassiana* isolates in each one of five wings of *Anopheles aquasalis*.

Beauveria bassiana	*Anopheles aquasalis*														
	1º Wing			2º Wing			3º Wing			4º Wing			5º Wing		
Isolate	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**
J80B	9	9	1	3	3	0	7	7	1	10	10	0	3	3	0
L46C	2	0	0	1	1	0	3	3	0	2	2	0	2	1	0
S6C	2	0	0	3	0	0	3	3	0	2	1	0	2	0	0
S52C	5	5	1	4	4	1	9	9	1	17	17	2	13	13	0
Boveril®	5	2	1	8	8	4	14	13	9	1	1	0	13	8	0
URPE-4	3	2	0	1	1	0	3	1	0	4	4	0	7	6	2
URPE-14	18	16	2	9	9	0	2	2	0	6	6	1	2	1	0

Number of conidia (C), number of germinated conidia (G) and number of appressoria formed (A) of *Metarhizium anisopliae* isolates in each one of five wings of *Anopheles aquasalis*.

Metarhizium anisopliae	***Anopheles aquasalis***														
	1º Wing			**2º Wing**			**3º Wing**			**4º Wing**			**5º Wing**		
Isolate	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**	**C**	**G**	**A**
C26A	16	16	3	2	1	0	2	2	1	10	5	1	2	0	0
C46A	9	9	3	13	13	2	4	4	2	9	9	2	8	8	2
C55A	24	0	0	14	1	0	0	0	0	43	3	0	11	0	0
C66A	7	7	3	32	26	13	2	2	0	9	7	3	5	2	1
C84A	3	3	0	6	6	2	5	5	0	0	0	0	1	0	0
C86C	12	0	0	26	0	0	20	4	0	5	0	0	16	0	0
J2B	23	20	5	25	22	5	23	23	7	27	26	4	12	12	3
J11B	29	25	7	11	11	4	24	22	0	5	5	0	30	26	2
J15D	15	12	5	20	17	3	23	22	8	7	5	2	30	24	7
J18A	18	14	5	7	7	4	0	0	0	4	4	0	2	2	1
J21C	8	7	0	3	2	0	8	7	4	8	8	2	2	2	0
J25A	1	1	0	9	3	0	2	0	0	3	1	0	2	0	0
J27A	1	1	0	3	3	0	18	18	6	10	10	3	4	4	0
J30B	26	23	7	2	1	0	1	1	1	14	14	6	1	1	0
J32A	4	4	1	7	7	2	7	7	3	7	7	0	1	1	0
J38A	15	0	0	2	0	0	2	0	0	2	1	0	1	0	0
J42A	4	1	0	2	2	0	0	0	0	4	4	2	1	0	0
J43A	5	4	0	8	8	2	2	1	0	16	16	2	6	6	2
J46A	6	0	0	2	0	0	1	0	0	3	2	0	6	0	0
J52B	8	8	3	11	7	2	3	0	0	1	0	0	1	1	1
J54B	9	8	4	1	1	0	1	1	0	9	9	3	4	3	1
J60A	6	6	0	27	25	6	18	18	8	3	3	1	5	5	4
J65C	6	5	5	17	0	0	67	14	5	14	6	2	31	27	2
J75A	30	1	0	4	2	1	25	20	0	2	0	0	7	6	0
J75B	1	0	0	1	1	0	11	10	3	45	33	3	11	8	5
J81C	40	40	8	14	14	5	17	10	3	7	0	0	5	5	1
L54A	7	7	1	3	3	1	2	0	0	9	5	1	7	7	0
L60A	3	3	1	27	24	13	3	3	3	14	13	9	2	2	1
Metarril®	2	2	1	2	2	0	1	1	0	1	0	0	1	1	0
URPE-11	3	3	0	3	3	1	7	6	3	12	10	9	12	12	4
URPE-19	9	9	2	1	1	0	11	11	6	4	4	2	11	11	7
URPE-30	7	4	0	8	8	0	18	18	0	8	8	0	2	2	0
URPE-32	0	0	0	7	7	1	5	4	1	3	3	2	1	0	0

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