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Screening of the Entomopathogenic Fungi, *Metarhizium anisopliae* and *Beauveri bassiana* against Early Larval Instars of *Anopheles stephensi* (Diptera: Culicidae)

[1]Mohammad Reza Fakoorziba, [2]Rahele Veys-Behbahani, [3]Navid Dinparast Djadid, [1]Kourosh Azizi and [4]Mona Sharififard

[1]Department of Medical Entomology and Vector Control, School of Health, Research Center for Health Science, Shiraz University of Medical Sciences, P.O. Box. 71645-111, Shiraz, Iran

[2]Department of Medical Entomology and Vector Control, School of Health and Student Research Committee, Shiraz University of Medical Sciences, P.O. Box. 71645-111, Shiraz, Iran

[3]Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran

[4]Department of Medical Entomology and Vector Control, School of Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Corresponding Author: Mona Sharififard, Department of Medical Entomology and Vector Control, School of Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT

Despite many prevention and control programs, the disease of malaria still remains a major health and economic problem in developing countries due to the unexpected resistance of malaria mosquitoes to chemical insecticides. In this study, the virulence of 10 Iranian isolates of the entomopathogenous fungi *Beauveria bassiana* and *Metarhizium anisoplaie* were evaluated against *Anopheles stephensi* larvae. Different strains were screened by adding aqueous suspension of 10^8 conidia mL^{-1} to 100 mL water containing 25 early instars larvae. The results showed that Bb 429C and Bb 796C were the most virulent isolates of *B. bassiana* causing 100% larval mortality with lethal times of 2.29 and 2.53 days for LT_{50} and 4.34 and 4.34 days for LT_{90}, respectively. Among *M. anisopliae* isolates, Ma 1018C was the most efficient isolate. Larval mortality rates caused by Ma 1018C at concentrations of 5×10^7, 10^8 and 5×10^8 conidia mL^{-1} were not significantly different as respectively they killed 99, 97 and 99% of larva at the fourth day. The lowest lethal times were related to the concentration of 5×10^7conidia mL^{-1} and were 0.63 and 1.93 days for LT_{50} and LT_{90}, respectively. Entomopathogenic fungi could be promising prospects and safe alternatives in integrated mosquitoes control programs.

Key words: Entomopathogenic fungi, *Metarhizium anisopliae*, *Beauveria bassiana*, *Anopheles stephensi*

INTRODUCTION

Despite the many prevention and control programs, malaria still remains a major health and economic problem in developing countries (WHO, 2010). The World Health Organization reported that 216 million cases of malaria resulting in 66,000 deaths occurred worldwide in 2010 (WHO, 2010, 2011). Approximately 60% of the populations of Eastern Mediterranean countries are in

endemic areas (with a high risk of malaria) and the Islamic Republic of Iran is one of these countries. Due to geographic conditions that restrict the transmission of malaria in Iran, the disease is in the elimination stage, although it is still endemic in the southern and southeastern provinces of Iran (Sistan-Baluchistan, Hormozgan and the tropical areas of Kerman) and included 95% of infectious cases in Iran (WHO, 2008; Haghdoost *et al*., 2008). The disease is transmitted by mosquitoes of the family Culicidae and *Anopheles stephensi* is one of the most important vectors of the disease in the Middle East and also in south and southeast Iran (Subbarao *et al*., 1987). Indoor Residual Spraying (IRS) and Insecticide Treated Nets (ITNs) were considered two effective techniques in mosquito control programs for many years. There is, however, a need to find and introduce effective alternative methods for the control of malaria vectors due to the unexpected resistance of this mosquito to some organochlorine, all organophosphorous and some pyrethroid insecticides (Enayati *et al*., 2003), as well as the high health risk for humans and also many environmental contaminations (N'Guessan *et al*., 2007; Moreno *et al*., 2008). The use of entomopathogenic fungi as biological control agents against agriculture pests has been greatly successful and their use in controlling mosquito larvae has recently been developed (Arthurs and Thomas, 2000; Blanford *et al*., 2005). *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metsch) Sorokin are the most common entomopathogenic fungal species. They reduce the *Anopheles* potential in malaria transmission by reducing female longevity, blood-feeding tendency and reproduction (Scholte *et al*., 2006). The conidia of these hyphomycete fungi can enter the body by penetrating the insect cuticle and is not necessarily eaten (McCoy *et al*., 1988). Since the virulence and growth ability of this parasite in the host body vary in different fungal strains, selection of the most virulence strain has a considerable impact on the effectiveness of a control program. Therefore, the objectives of this study were to screen 10 Iranian strains of *B. bassiana* and *M. anisopliae* against *A. stephensi* larvae.

MATERIALS AND METHODS

***A. stephensi*:** This study was done using a colony of a susceptible strain of *A. stephensi* reared in the national insectarium of the Malaria Research Center at the Pasteur Institute of Iran. All rearing steps were performed under 27±1°C, 70±5% Rh and a photoperiod of 12:12 (L:D). Adults were fed with 10% glucose and the guinea pig was selected for female blood meals. The larvae were fed with a mixture of dog food and yeast (3:1). Eggs were collected and kept in plastic containers containing water (22-25°C) until they become larva. After the larvae became pupae, the pupae were removed daily and placed into the adult cages. A paper cone was placed on each pupa cup to prevent adults from entering the cups and newly emerged adults were released to the cage. The life cycle of *A. stephensi* is 10-12 days under these conditions.

Entomopathogenic fungi: Ten Iranian strains of *B. bassiana* and *M. anisopliae* were provided by the Plant Protection Institute of Iran. They were cultured on Sabouraud Dextrose Agar with Yeast Extract (SDAY) and kept at 27°C, 75±5% Rh and a photoperiod of 12:12 (L:D). The dry conidia were removed from the surface of the culture plate with a scalpel and transferred to sterile distilled water containing 0.01% Tween-80. The concentration of the suspension was determined using a hemocytometer.

Experiment 1

Regain fungal virulence: In order to regain the virulence of fungal strains, they must be passed through an appropriate host (Butt and Goettel, 2000). So, the larval stage of the housefly, *Musca domestica* L. was selected for passage in this study due to its short life cycle, ease of rearing

and the appearance of muscardine symptoms. Aqueous suspension of each fungal strain was prepared and groups of 20-25 larvae (instar 2) were immersed in each conidial suspension for 10 sec. Then they were placed on damp filter paper for at least 12 h in order to increase the probability of conidia penetration in the host body. After that, the larvae were transferred to bedding. Larval cadavers were removed daily and the surface was sterilized by 10% sodium hypochlorite solution for 2-3 sec, rinsed in sterile distilled water 2-3 times and then kept in sterile Petri dishes covered with damp filter paper until sporulation. The sporulation cadavers were held at 4°C, for use in subsequent cultures (Sharififard *et al*., 2011).

Experiment 2

Fungal strains screening: To obtain the required volume of conidia with high virulence, sporulating larvae of the housefly obtained from 10 strains of *B. bassiana* and *M. anisoplae* were separately cultured on SDAY. After 14 days, the aqueous suspension of each fungal strain was prepared using Tween-80 (0.01%) and the concentration was adjusted to 10^8 conidia mL^{-1}. Twenty-five first and second-instar larvae were added to each disposable cup containing 100 mL distilled water. One milliliter of each conidial suspension was added to each cup. The containers were kept under conditions of 27±1°C, 70±5% Rh and a photoperiod of 12:12 (L:D). The number of larvae that died or pupated was recorded daily for the following 10 days. The experiments were replicated four times. Another 4 replicates of 25 untreated larvae were maintained as controls. The larvae were provided with cat biscuits as food every two days.

Experiment 3

Virulence evaluation of *M. anisoplaie* Iran 1018C strain: In order to determine the pathogenicity of the most virulence isolate of *M. anisopliae* against *A. stephensi* larvae, stock suspension with a concentration of 10^9 conidia mL^{-1} was prepared. Serial dilution method with $C_1V_1 = C_2V_2$ equcation was used for preparation of low concentrations (5×10^8, 10^8, 5×10^7 and 10^7). After that, 1 mL of each concentration was added to containers each containing 100 mL distilled water and 25 first and second instar larvae. The experiment was replicated 4 times. The containers were kept under conditions previously mentioned.

Data analysis: The mortality data were corrected by Abbott's equcation (Abbott, 1925). Analyses of variances (ANOVA) and comparison of the means of mortality percentages were done in a completely randomized design by Tukey´s test ($p<0.05$), using SAS software (version 9.1.3). In order to determine the lethal concentration (LC_{50}) and lethal time values of each concentration (LT_{50}), the data were submitted to the probit analysis model of the SAS software. When there was no overlap in the 95% CL of lethal time values, the differences in treatments were considered significant.

RESULTS

The pathogenicity of 6 strains of *B. bassiana* and 4 strains of *M. anisopliae* was different at 3 and 7 days after exposure ($p = 0.05$) (Table 1, 2). Although all *B. bassiana* strains caused mortality in *A. stephensi* larvae and their pathogenicity differed with that of the control group, Bb 429C and Bb 796C were, respectively, the most virulent strains with 65% and 63% mortality at 3 days post-exposure (Table 2). Larval mortality rates, due to *B. bassiana*, increased in the following days and reached 100% in Bb 429C and Bb 796C on the seventh day. The lethal times

Table 1: Fungal isolates used in bioassays against *Anopheles stephensi* larvae

Site and date of origin	Insect host or substrate	Isolates	Species	Fungi
Ardebil- 1995	*Leptinotar sadecenlineata*	IRAN 187C	*Bassiana* (Bals.) Vuill	*Beauveria*
Gilan-Rasht 2001	*Chilosuppressalis* Walker.	IRAN 428C		
Hassan rud 2001	*C. suppressalis* Walker.	IRAN 429C		
Karaj-Atashgah 2001	Soil	IRAN 440C		
Urmia 2004	*Heterodera schachtii*	IRAN 796C		
Urmia 2004	*H. schachtii*	IRAN 797C		
Mazandaran	*C. suppressalis* Walker.	IRAN 437C	*Anisopliae* (Metschn.) Sorokin	*Metarhizium*
Rasht	*Parandracaspica* (Col.)	IRAN 1018C		
	Locust	IRAN 715C		
Saravan	*Rhyncophorus ferrugineus*	DEMI 001		

Table 2: Mortality means and lethal time values (after 7 days) of early instar larvae of *A. stephensi* exposed to 10^8 conidia mL^{-1} of *B. bassiana and M. anisopliae*

LT_{90} (C.I. 95%)	LT_{50} (C.I. 95%)	Mortality Means %±SE *(after 7 day)*	Mortality Means %±SE *(after 3 days)*	Fungal strain
3.82 (3.61-4.12)	2.77 (2.64-2.89)	100.0	60.0 (±6.6)	Ma 1018 c
4.09 (3.88-4.39)	2.87 (2.74-3.01)	100.0	41.5 (±9.1)	Ma 437 c
4.05 (3.83-4.34)	2.81(2.65-2.98)	100.0	48.3 (±2.1)	Ma R c
4.35 (4.13-4.64)	3.15 (3.02-3.28)	100.0	44.3 (±7.7)	Ma 715 c
8.93 (8.12-10.33)	6.03 (5.77-6.35)	66.0 (±8.8)	9.5 (±3.1)	Bb440 c
8.40 (7.78-9.40)	6.00 (5.77-6.26)	74.4 (±11.1)	5.5 (±5.5)	Bb428 c
7.99 (6.39-17.2)	5.41 (4.31-7.01)	81.2 (±9.8)	4.8 (±3.4)	Bb797 c
4.34 (3.87-5.05)	2.29 (2.11-2.48)	100.0 (±0.00)	65.0 (±1.00)	Bb429 c
4.34 (3.91-5.0)	2.53 (2.35-2.71)	100.0 (±0.0)	63.0 (±4.4)	Bb796 c
-	-	26.7 ((±4.1)	6.00 (±2.5)	Bb187 c

Table 3: Mortality percentage Means (±SE) of *A. stephensi* exposed to of *M. anisopliae* Iran 1018C

Concentrations (conidia mL^{-1})	Mortality Means%(±SE) (24 h)	Mortality Means%(±SE) (48 h)	Mortality Means%(±SE) (72 h)
107	20.5 ± 1.3^{e}	51 ± 3.4^{c}	91 ± 3.4^{ab}
5×10^7	60.0 ± 5.6^{a}	91 ± 1.9^{a}	99 ± 1.0^{a}
10^8	61.0 ± 1.0^{a}	81 ± 1.9^{ab}	97 ± 1.9^{ab}
5×10^8	18.0 ± 1.1^{b}	69 ± 5.0^{b}	99 ± 1.0^{a}
109	20.0 ± 2.8^{b}	32 ± 2.8^{d}	84 ± 5.4^{b}
^{c}ontrol	0.0 ± 0.0^{c}	0^{e}	0^{c}

*Dissimilar letters in each column indicate significant differences

of *A. stephensi* larvae by these two strains were 2.29 and 2.53 days for LT_{50} and 4.34 and 4.34 days for LT_{90}. There were no significant differences between the lethal times of Bb 429C and Bb 796C because their confidence limits completely overlapped. Other *B. bassiana* strains also caused high mortality in the early larval instars but it took long time. In other words, the highest mortality rates in the lowest lethal times were related to Bb 429C and Bb 796C (Table 2). The pathogenicity of *M. anisopliae* strains were also different and Ma 1018C was the most virulent strain due to its higher mortality at a shorter lethal time (60 and 97% mortality at 3 and 7 days) (Table 2). The differences in lethal times and the eventual mortality rates of Ma 437C, Ma 1018C and Ma DEMI 001, however, do not appear to be significant (Table 2).

In the second bioassay test, *M. anisopliae* Iran 1018C caused varying mortality rates in the early larval instars of *A. stephensi* at different concentrations and times (Table 3). Mortality rates increased with increased conidia concentrations in Ma 1018C from 10^7 to 5×10^8 conidia mL^{-1} but

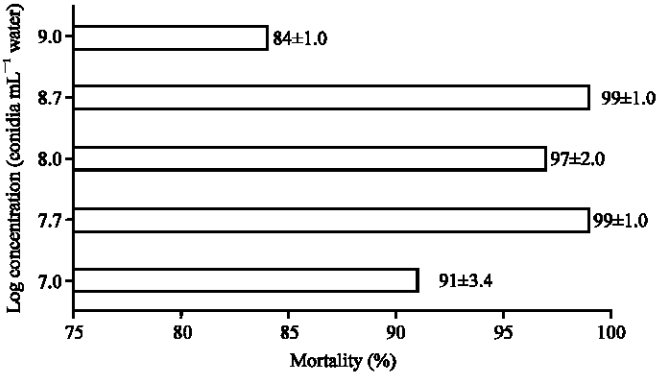

Fig. 1: Mortality percentage means (±SE) of *A. stephensi* exposed to different concentrations of *M. anisopliae* Iran 1018C after 72 h

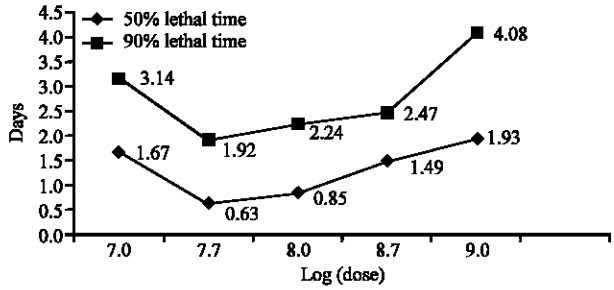

Fig. 2: Lethal time values (day) of *M. anisopliae* 1018C against early instar larvae of *A. stephensi*

it decreased in the concentration of 10^9 conidia mL^{-1} (Fig. 1). There were no significant differences in concentrations of 5×10^7, 10^8 and 5×10^8 conidia mL^{-1} (p-value = 0.05) and they, respectively killed 99, 97 and 99% of larva on the fourth day. Comparison of lethal time values of Ma 1018C at five concentrations cleared no uniform increase or decrease trend as the lethal time decreased from 10^7 to 5×10^7 and increased again in 10^8 to 10^9 conidia mL^{-1}. The lowest lethal times were related to the concentration of 5×10^7 as they were 0.63 and 1.93 days for LT_{50} and LT_{90}, respectively (Fig. 2). The mortality rate in this concentration, however, was equal to the mortality rate observed in 5×10^8 conidia mL^{-1} (99%) but the lethal time values in the concentration of 5×10^7 conidia mL^{-1} were lower than the previous one. We were unable to determine the lethal concentrations (LC_{50} and LC_{90}) probably because the mortality rates showed no significant difference and were all above 50%.

DISCUSSION

Fungal diseases in insects are common and widespread and can decimate their populations in spectacular epizootics. Nearly all insect orders are susceptible to fungal diseases, including dipterans. Fungal pathogens such as *Lagenidium*, *Coelomomyces* and *Culicinomyces* are known to affect mosquito populations and have been studied extensively. There are, however, many other fungi, such as *Beauveria* and *Metarhizium* that infect and kill mosquitoes at the larval and/or adult stage (Scholte *et al*., 2004). Problems such as mass production, storage conditions and the high dosage required to kill mosquitoes were reasons that stopped researchers from applying these microbial agents as alternatives for chemical insecticides.

To enhance the potential of this microbial control agent, improvements should be made to develop new culture media, economic storage conditions that can prolong the stability of infective stages and to develop formulations that increase the likelihood of contact with target species, such as flotation in *Anopheles* habitats. Certain formulations can improve the ability of entomopathogenic fungus spores to spread over a water surface as well as increase their persistence and can lead to an enhanced efficacy of fungal spores (Bukhari *et al*., 2011). Successive subculturing on artificial media often results in a decrease in virulence. The virulence of attenuated isolates can often be regained, however, by passing through an appropriate host. The effects of culture history on virulence poses a special problem if bioassays are used to compare virulence among isolates obtained from various sources and culture collections. In an attempt to address this problem, each isolate can first be passed through an insect host prior to culture on artificial media and use in bioassays (Butt and Goettel, 2000). For this purpose, the larval stage of the housefly, *Musca domestica* L. was selected for passage of the selected fungal strains in this study and the new cultures were used in screening tests against *A. stephensi* larvae.

Many previous studies demonstrated the efficacy of *Beauveria* and *Metarhizium* against adults of *Anopheles* spp. (Scholte *et al*., 2006; Kannan *et al*., 2008; Mnyone *et al*., 2010; Mouatcho, 2010; Farenhorst *et al*., 2007) but there is little research (Alves *et al*., 2002; Bukhari *et al*., 2011; Prasad and Veerwal, 2010) about the use of these agents to control larvae in their water habitats. The results of our study detected that almost all the selected Iranian isolates of *B. bassiana* and *M. anisopliae* were pathogenic against first and second instar larvae of the *A. stephensi* strain Chabahar and larval mortality was considerable at 4-7 days after exposure. The pathogenicity of these entomopathogenous fungi against larvae of *A. stephensi* and *A. gambiae* was also reported by Bukhari *et al*. (2011). Our results also confirmed those of (Prasad and Veerwal, 2010) who observed 23-61% mortality in *A. stephensi* larvae using *B. bassiana* conidia. In another study, the mortality rate of *Culex quin quefaciatus* larvae was reported as 0-90% at 5 days after using *M. anisoplaie* (Alves *et al*., 2002; Lacey *et al*., 1988). This study also confirms our observations that these fungi are pathogenesous against mosquito larvae in their water habitat. Additionally, all the selected Iranian strains of *B. bassiana* and *M. anisopliae* were virulent against the housefly, *M. domestica,* as they caused 28-100% mortality in larvae and adults (Sharififard *et al*., 2011).

We selected the *M. anisoplaie* Iran 1018C strain for further evaluation because it caused a high mortality in a lower lethal time in preliminary screening tests. Larval mortality rates did not show a proportional increase or decrease with an increase in spore concentrations, as the mortality rate was lowest at the highest concentration of 10^9 conidia mL^{-1}. At a certain concentration, however, it increased with time. Fungal spores are hydrophobic and they clump together into masses when applied over the water surface without a suitable surfactant, so spore feeding by larvae or spore attaching to larval cuticle will be reduced (Prasad and Veerwal, 2010). We used Tween-80 as a surfactant and since the spores of *Metarhizium* do not clump together like or as much as *Beauveria,* the decreasing rate of mortality in the highest concentration could be the result of clumping or due to the sedimentation rate of spores. In higher concentrations these two reasons can result to avoid spore attachment to the larval cuticle. All at all, *M. anisopliae* has several characteristics that make it interesting as a microbial mosquito control agent. It causes high mortality of mosquito larvae in laboratory populations, the fungus can be grown in massive amounts on inexpensive artificial media and conidia can be stored easily. Moreover, its failure to germinate in the mosquito environment until actual exposure to a host and its resulting persistence in the environment as well as the fact that its effect is not limited to periods of host molting (as for *B. bassiana*) make this

fungus a very promising control agent. *B. bassiana* conidia germinate in mosquito habitats even when not in contact with larvae. This limitation along with the high dosage needed is serious drawbacks for mosquito larval control (Scholte *et al*., 2004). Our results were obtained under controlled conditions and on a small scale. It is necessary, however, to evaluate the table *M. anisoplaie* and *B. bassiana* under field conditions and on a large scale. It is also necessary to evaluate other formulations of the selected isolate such as conidia-dust formulation and oil formulation against *A. stephensi* larvae.

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