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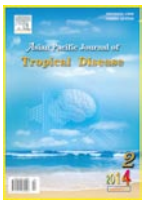

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Laboratory evolution of the entomopathogenic fungus *Beauveria bassiana* against *Anopheles stephensi* larvae (Diptera: Culicidae)

Rahele Veys-Behbahani[1], Mona Sharififard[2], Navid Dinparast-Djadid[3], Javad Shamsi[2], Mohammad Reza Fakoorziba[1*]

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

[2]*Department of Medical Entomology and Vector Control, School of Health, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran*

[3]*Malaria and Vector Research Group, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran*

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ABSTRACT

Objective: To examine *Beauveria bassiana* (*B. bassiana*) fungus bioassay against the larval stages of *Anopheles stephensi* in Iran.

Methods: The fungal suspension by the concentrations of 1×10^9, 5×10^8, 10^8, 5×10^7 and 1×10^7 conidia per milliliter have been prepared in different volumes (2, 4 and 6 mL) and each concentration were added to containers containing 25 *Anopheles* larva instars 1 and 2. The mortality of the dead larvae with abnormal symptoms was recorded as a result of the fungal infection after 24, 48 and 72 h.

Results: Comparison between the mean mortality rate of *Anopheles stephensi* larva at different concentrations of *B. bassiana* strain Iran 429C at 2, 4 and 6 mL showed that there was no significant relation of the mean mortality rate of larvae at concentrations of 1×10^9 and 5×10^8, and after 48 h resulted in 100% mortality rate of the larvae populations. In addition, there is no significant differences in the amounts of lethal times (LT) (LT_{50} and LT_{90}) as LT_{90} values calculated at a concentration of 5×10^8 and in volumes 2, 4 and 6 mL were 1.46, 1.36 and 1.08 d, respectively.

Conclusions: *B. bassiana* strain Iran 429C in 2 mL of 5×10^8 concentration or the concentration of a 1×10^9 mL per 100 mL of water is recommended as the optimal concentration for the control of *Anopheles* larvae. The development of suitable formulations of entomopathogenic fungi may be a promising prospect in the mosquito control programs.

1. Introduction

The positive impact of using indoor residual spraying and insecticide-treated bed net, the most common methods of controlling the mosquito vectors of malaria in the world, has been well illustrated. Nevertheless, World Health Organization annual report on creating different types of vector resistance to synthetic insecticides suggests the need to develop alternative ways to achieve the goal of eliminating malaria[1,2]. *Anopheles stephensi* (*An. stephensi*) mosquitoes, which are the main malaria vector in the Middle East, including Iran, have shown a relative resistance to insecticides such as DDT and tolerance to deltamethrin, bendiocarb and fenthion larvicide[3].

Recently, biological control methods such as using entomopathogenic fungi have shown that these factors potentially involved in the control of many species of mosquitoes at different stages[4-6]. *Beauveria bassiana* (*B. bassiana*) is one of the most important entomopathogenic fungi, studies on this fungus have demonstrated a positive impact of *B. bassiana* on the larvae and adult mosquitoes[7-9]. This fungus is in the hyphomycetes category easily removed by simple and inexpensive synthetic medium from the insect's bodies and soil[10].

The fungi conidia do not affect non-target organisms, but they can contaminate the host by contact without being consumed. Consequently, the larvae are easily exposed and infected when they meet the water[11]. In our previous study,

*Corresponding author: Prof. Dr. Mohammad Reza Fakoorziba, Department of Medical Entomology and Vector Control, School of Health, Shiraz University of Medical Sciences, P.O.Box 71645-111, Shiraz, Iran.
Tel: +9871 1725 1020; +9891 7711 3112
Fax: +9871 1726 0225
E-mail: fakoorziba@sums.ac.ir; mrfakoor@yahoo.com

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the effect of 10 Iranian isolates of *B. bassiana* fungus to control instars 1 and 2 larvae were demonstrated *in vitro*[12]. The purpose of this study was concluded to ascertain the effective volume and concentration of the strains that had the greatest impact on the mortality rate of the instars 1 and 2 larvae.

2. Materials and methods

2.1. Rearing of An. stephensi

This study was done using susceptible strain of *An. stephensi* colony reared in national Insectarium in Malaria Research Center at the Pasture Institute of Iran. All rearing steps were under (27±1) °C, 70%±5% relative humidity and photoperiod of 12:12 (light: dark). The adults were fed with 10% glucose and also guinea pig was selected for female taking blood meals. The larva was fed with 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 and placed into the cage of adults daily. A paper cone was placed on each pupa cup to prevent adults from entering into the cups and newly emerged adults can be released to cage. The life cycle of *An. stephensi* takes 10–12 d under these conditions.

2.2. Determain and culture of entomopathogenic fungi

B. bassiana strain Iran 429C was determined as the most pathogenic isolate against *An. stephensi* larva in the preliminarily screening tests[12]. It was cultured on sabouraud dextrose agar with yeast extract and kept at 27 °C, 75%±5% relative humidity and photoperiod of 12:12 (light: dark). The dry conidia were removed from the surface of the culture plate with a scalpel and transferred them to sterile distilled water containing 0.01% Tween-80. The concentration of the suspension was determined using a hemocytometer.

2.3. Virulence evaluation of B. bassiana strain Iran 429C

In order to determine pathogenicity of *B. bassiana* strain Iran 429C against *An. stephensi* larva, stock suspension with concentration of 10^9 conidia/mL was prepared. Serial dilution method with $C_1V_1=C_2V_2$ formula was used for preparation of low concentrations (5×10^8, 1×10^8, 5×10^7 and 1×10^7). After that, 6, 4 and 2 mL of each concentration were added to containers each containing 100 mL distilled water and 25 1st and 2nd instars larvae. The experiment was replicates 4 times. The containers were kept under conditions previously mentioned.

2.4. Data analysis

The mortality data were corrected by Abbott's formula[13]. ANOVA and comparison 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 lethal time (LT) values of each concentration (LT_{50} and LT_{90}), the data were submitted to Probit analyses model of SAS software. When there was no overlap in the 95% confidence interval (*CI*) of LT values, the treatments difference were considered significant.

3. Results

Comparison of mortality of instars 1 and 2 larvae of *An. stephensi* at different concentrations of the fungus *B. bassiana* strain Iran 429C at volumes of 2, 4 and 6 mL of each was surveyed. The concentrations showed that there is a significant relation between mortality of larvae in different concentrations of fungi (1×10^9, 5×10^8, 1×10^8, 5×10^7, 1×10^7 conidia/mL) with a certain volume compared with the control group. ($P\leq0.05$, $df=5$). With increasing concentration of conidia suspension for a specified volume, the amount of larval mortality increases as the mean concentrations of 1×10^9 and 5×10^8 conidia/mL showed 100% mortality in the three volumes used (Table 1).

Table 1
Percentage of mortality in different concentrations of *B. bassiana* Iran 429C fungus in 2, 4 and 6 mL of a fungal suspension against instars 1 and 2 larvae of *An. stephensi*.

Concentrations (Conidia/mL)	%Mortality transformed		
	6 mL	4 mL	2 mL
1×10^7	13.3 ± 7.3^D	8.9 ± 1.4^D	2.9 ± 5.4^D
5×10^7	41.8 ± 3.0^C	39.5 ± 8.2^C	24.7 ± 5.3^C
1×10^8	84.3 ± 4.5^B	82.5 ± 4.3^B	57.0 ± 4.5^B
5×10^8	100.0 ± 0.0^A	100.0 ± 0.0^A	100.0 ± 0.0^A
1×10^9	100.0 ± 0.0^A	100.0 ± 0.0^A	100.0 ± 0.0^A
Control	0.0 ± 0.0^E	0.0 ± 0.0^E	0.0 ± 0.0^E

All values are expressed as mean±SE; A–D : Values with same letters have no significant difference.

The comparison between the mortality of larvae at different volumes (2, 4 and 6 mL) at a specified concentration of fungal suspension showed that there is a significant difference between volumes of a specified concentration and the control group ($P\leq0.05$, $df=3$). Larval mortality increased with the increased volume of conidia suspension, as mortality at the concentration of 1×10^7 conidia reached from of 2.9%±5.4% at 2 mL to 13.3%±7.3% at 6 mL. Also, at the concentration of 5×10^7, reached from 24.7%±5.3% at 2 mL to 41.8%±3.0%, and at 1×10^8 concentration reached from 57.0%±4.5% at 2 mL to 84.3%±4.5% at 6 mL. Furthermore, all three used volume concentrations of 1×10^9 and 5×10^8 resulted in 100% larval mortality in a population of *An. stephensi* instars 1 and 2 larvae (Table 1).

The time required to eliminate 50% of the population (LT_{50}) and 90% of the population (LT_{90}) of larval population was reported. By increased concentrations, LT_{90} and LT_{50} rates

decreased. Concentrations of 1×10^9 and 5×10^8 in various volumes caused 50% mortality in the larval population at less than 24 h, and 90% mortality of the larvae in less than 48 h. A comparison of LT_{50} and LT_{90} about assurances at 2 and 6 mL of 5×10^8 concentration showed no significant difference in these values. The maximum amounts of LT_{50} and LT_{90} were observed at 1×10^7 and volume of 4 mL at 6.26 d and 16.38 d, respectively. Also in 2 and 6 mL volumes of this concentration, mortality was observed after 7 d was less than 50%, so it is not possible to calculate LT_{50} and LT_{90} (Table 2).

Table 2

LT_{50} and LT_{90} at different concentrations of *B. bassiana* at 2, 4 and 6 mL volumes of fungal suspension against larvae of *An. stephensi*.

Concentration (Conidia/mL)	Volume (mL)	LT_{50} (d) (95% *CI*)	LT_{90} (d) (95% *CI*)
1×10^9	2	0.95	1.09
	4	0.91	1.05
	6	0.91	1.05
5×10^8	2	0.97 (0.87-1.05)	1.46 (1.31-1.75)
	4	0.94	1.36 (1.23-1.64)
	6	0.86 (0.71-0.95)	1.08
1×10^8	2	2.40 (2.06-2.82)	9.04 (6.43-16.5)
	4	1.41 (1.21-1.59)	3.53 (3.05-4.35)
	6	1.25 (0.02-2.00)	3.20
5×10^7	2	4.89 (3.22-8.96)	16.42 (8.96-19.7)
	4	2.81	7.92
	6	2.80	8.21
1×10^7	2	-	-
	4	6.26	16.38
	6	-	-

4. Discussion

Not only the appearance of chemical larvicides resistance but resistance to the most common biological control agents *e.g. Bacillus sphaericus* and *Bacillus thuringiensis* demonstrates the importance of alternative methods for controlling mosquitoes[14,15].

Today the use entomopathogenic fungal biological control agents of mosquitoes are very important. Studies show that the impact of the fungus *B. bassiana* on different life stages of mosquitoes varied. The fungus has not been effective against *Aedes aegypti* larval stages, and *Ochlerotatus sierrensis* and the larvae of *Culex quinquefasciatus*[5,10,11], while it caused 100% mortality after 5 d in adult *Aedes aegypti*, *Ochlerotatus sierrensis*[10]. The effect of the fungus on the larvae and adult *Anopheles* mosquitoes are shown to be well[12,16-18].

Studies have shown that a variety of factors such as the stage of larvae, fungus variety, and various concentrations of fungus could be effective in increasing the susceptibility of the host to the fungus[19]. For example, the younger age of instars 1 and 2 larvae are more possibly infected fugally due to a longer growth time to the reach pupation stage, therefore, a better possibility to make contact with fungus spores or by feeding on them. Moreover, their cuticle is softer than the 3rd and 4th instars larvae, allowing the fungus to infiltrate through the cover, which increases the cuticle contact with fungus[19]. This study shows that with increased concentrations and contact times of *B. bassiana* Iran 429 C conidia strain, the mortality of instars 1 and 2 larvae of *An. stephensi* increases, which may be due to increased fungus conidia consumed by host as well as increased cuticle contact with fungus conidia[12]. Moreover, increased volumes of various concentrations show a reasonable increase of mortality rate. Earlier pathogenic property of the fungi against *An. stephensi* larvae and *Anopheles gambiae* also showed that the percentage of fungus carriers this larvae was reduced to 39%-50%[7]. The results are similar to Prasad and Veerwal that their findings show 23%-61% mortality at a concentration of 6.4×10^{11} against *An. stephensi* larvae[20], but there was no reasonable increase in larval mortality with the increasing volume of fungus conidia. Because the spores are hydrophobic and when used on the water surface without the use of surfactants clumps are created and the larvae do not accept them as food. Another study shows the efficacy of *B. bassiana* Iran 429C against *Musca domestica* as causing 86% larvae mortality and 94% adult mortality[21].

The larvae shapes infected with the fungus were altered, these changes augmented with increased contact time with the fungus conidia, with more tangible signs. First, cuticle was destroyed with black pigments on some parts. Then head, neck, end parts of the body and later digestive organs have transformed with black pigments in larvae body. All of these transformation caused due to fungus spores entrance, their reproduction, hyphae and related toxins creation. Moreover, since the infection transmission from larvae to pupe and adult by fungus is demonstrated, in this study there were some larvae that were not destroyed upon contact with the fungus. The larvae that reach to instars 3, displayed infection signs and transformed into a stage 4 before pupation, they never become pupae, and these observations have been reported by Miranpuri and also Prasad[20,22]. Additionally, Sharififard (2011) showed that *B. bassiana* fungal infection caused deformation of the dead larvae. The accumulation of hardened mycelium originally changed to milky white, next pink and then purple with muscardine marks that found on the body of dead larvae[21].

There were no significant differences in the mean mortality rate of the larval *An. stephensi* in concentrations of 5×10^8 and 1×10^9, both of bulk concentrations causing 100% mortality of the larvae in less than 48 h. In addition, a comparison of assurance values of LT_{50} and LT_{90} at different volume concentrations of 5×10^8 also indicates no significant difference. Therefore, the concentration of 5×10^8 with a volume of 2 mL (*i.e.* 1 mL of 1×10^9 concentrations in 100 mL water) is recommended as the optimum concentration for larval control. Further research should be continued to analyze proposed isolation in the natural conditions and

in a wider scale with new formulations, to determine the best concentration and volume of the fungus conidia. This study indicated the necessity of studying the synergistic or antagonistic isolates as well as investigating the relationship between pesticides that are commonly used to control *An. stephensi*.

Conflict of interest statement

We declare that we have no conflict of interest.

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