Entomology, Ornithology & Herpetology

Vyas et al., Entomol Ornithol Herpetol 2015, 4:4
DOI: 10.4172/2161-0983.1000162

Research Article Open Access

Larvicidal Activity of Metabolites of *Metarhizium anisopliae* against Aedes and Culex Mosquitoes

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Abstract

The objective of this study was to determine the larvicidal effects of entomopathogenic fungus *Metarhizim anisopliae* against degue, chikunguniya and filariasis disease vectors. The fungus was cultivated in the complete broth media and the extracelluar metabolites were filtered by using Whatman no.1 filter paper. Further, the filterd metabolites were conducted for its larvicidal efficacy against all instars of *Ae. egypti and Cx. quenquefacistus,* at five different significant concentrations (2.35, 2.65, 2.83, 2.95 and 3.05ppm). Larvae of *Cx. quenquefacistus* were found more susceptible than larvae of *Ae. aegypti*. The hightest LC_{99} value (663.74ppm) was resulted in the first instar of *Cx. quinquefasciatus* while the lowest LC_{99} value (309.02ppm) was found in third instar of *Ae. aegypti*. The findings of this preliminary study gives overview idea about the different larvicidal properties of the metabolites of *M. anisopliea.* Additionally it will help us to find specific larvicidal compound for mosquito borne disease control applications.

Keywords: Entomopathogenic fungi; *Metarhizium anisopliae*; *Aedes aegypti*; *Culex quinquefasciatus*

Introduction

Culex and Aedes are major vectors of zoonotic diseases in tropics. It causes morbidity of millions of persons resulting in loss of man-days causing economic loss [1]. *Culex quinquefasciatus*, a vectore of lymphetic filariasis, is widely distributed. On the other hand, *Aedes aegypti* is a vector of dengue, chikunguniya, yellow fever and that carries the arbovirus responsible for these diseases is also widely distributed in the troical and subtropical zones. In Indian scenario, almost the entire country is endemic to the mosquito borne diseasses due to favorable ecological conditions. To prevent mosquito-borne diseases and improve public health, it is necessary to control them. Mosquito in the larval stages are attractive target for pesticides because mosquitoes breed in water, and thus it is easy to deal with them in this habitat.

Numerous chemical larvicides are known to have toxic effects beyond their target pests including toxic effects to animals and human. The opportunity to substitute safer, more selective and biodegradable biocontrol agent can provide important ecological benefits. The use of microbial larvicides could decrease our dependence on chemical insecticides. The entomopathogenic fungus life cycle is associated with the synthesis and secretion of different active metabolites, including extracellular enzymes and low molecular weight compounds (toxins). These toxic byproducts mainly help the organisms to withstand and protect themselves from invading pathogens [2,3]. In general fungi produce a wide range of secondary metabolites with diverse biological activites like antibiotics, cytotoxic substances, insecticides compound that promote or inhibit growth, attractor, repellent etc.[4]

Entomopathogenic fungal metabolites could be an alternative source for mosquito larvicides because they constitute a potential source of bioactive compounds and generally free from harmful effects. Moreover, use of these microbial larvicides in mosquito control instead of synthetic insecticides could reduce the cost and environmental pollution. The aim of present investigation is to assess the comparative efficacy of extracellular metabolites of *Metarhizium anisopliae* against all four larval stages of *Ae. aegypti* and *Cx. quinquefasciatus* in the laboratory.

Material and Methods

Microbial culture

The fungal strain of *M. anisopliae* (MTCC-892) was procured from the Institute of Microbial Technology, Chandigarh, India. Fungal colonies were cultured in 250 ml conical flask containing 100ml of complete broth media (0.001g $FeSo_4$, 0.5g KCL, 1.5g KH_2PO_4, 0.5g $MgSo_4 7\ H_2O$, 6g $NaNO_3$, 0.001g $ZnSO_4$, 1.5g Hydrolyzed Casein, 0.5g Yeast Extract, 10g Glucose, 2g Peptone and 1000ml Deionized water) fungi were incubated under static condition 25 ± 2^{0}C for 15 ± 2 days with constant aeration in BOD.

Maintenance of mosquito larvae in the laboratory

The colonies of *Ae. aegypti* and *Cx. quinquefasciatus* were maintained in the laboratory at a temperature of 25 ± 2°C, relative humidity was 75 ± 2% and photoperiod of 14:10 (L/D).

Filtration of extracellular metabolites

Cell free culture filtrates were obtained by filtering the broth through successive Whatman No.1 filter paper after incubation. Fungal metabolites present in the filtrate were used to examine the larvicidal activities.

Bioefficacy

Mosquito larvae of *Ae. aegypti* and *Cx. quinquefasciatus* were separated and placed in separate containers (60cm × 40cm × 20cm), containing microbe free Deionized water. After that, different test

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Received August 01, 2015; **Accepted** August 18, 2015; **Published** August 24, 2015

Citation: Vyas N, Dua KK, Prakash S (2015) Larvicidal Activity of Metabolites of *Metarhizium anisopliae* against Aedes and Culex Mosquitoes. Entomol Ornithol Herpetol 4: 162. doi:10.4172/2161-0983.1000162

concentrations of metabolites in 100ml water were prepared in 250ml beakers. Bioassays were conducted separately for each instar of mosquitoes at five selected log concentrations. Log concentrations for *M. anisopliae* were 2.35, 2.65, 2.83, 2.95 and 3.05ppm. To test the larvicidal activity, 20 larvae of each stage were separately exposed to 100ml of test concentrations. Similarly, the control was run to test the natural mortality, except concentrations of culture medium used instead of the fungal filtrates [5].

Mortality and survival were recorded after 24, 48 and 72h of the exposure. During experimental time, no food was offered to the larvae. The experiments were replicated thrice to validate results. All test containers were tightly covered with pierced aluminium foil and placed at room temperature without sunlight.

Data management and statistical analysis

The efficacy study of filtrate metabolites of *M. anisopliae* were assessed against larvae of *Ae. aegypti* and *Cx. quinquefasciatus*. Experimental test that demonstrated more than 20% control mortality were discarded and repeated when control mortality ranging between 5-20% was observed, and was corrected by Abbott's formula [6,7]. The concentrations produce 50%, 90% and 99% mortality in larvae (LC_{50}, LC_{90} and LC_{99} respectively) were calculated with their fiducial limits at 95% confidence level. The relation between probit equation and probit regression lines were drawn for each of the larval stages (Figures. 1 and 2).

Results

All instar of the *Ae. aegypti* and *Cx. quinquefasciatus* appeared to be susceptible to the crude metabolites of *M anisopliae*.

LC values and chi-square analysis for *Cx. quinquefasciatus* larvae

The estimates of LC_{50}, LC_{90} and LC_{99} values for *M. anisopliae* against *Cx. quinquefasciatus* have shown with their fiducial limits and probits equations (Table 1). It has shown that LC values were higher for the first instar than other instars. The probits were plotted against respective log concentrations and strait lines were drawn by eye to fit the points for each instar (Figure 1). The LC values for third instar

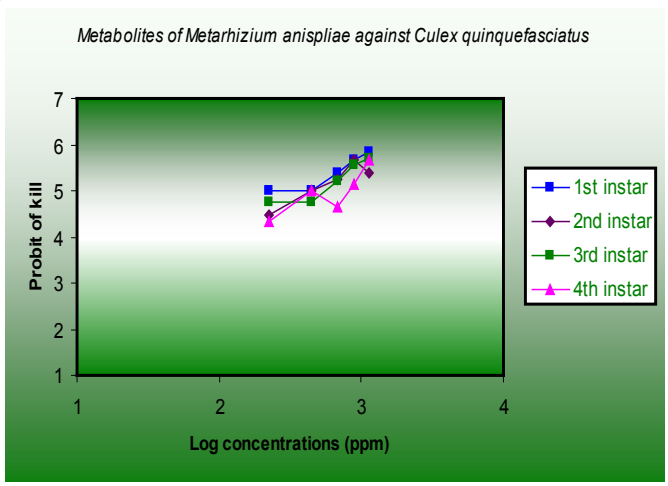

Figure 1: Comparative relationship between probit of kill and log concentrations of M. anisopliae filtrate metabolites showing probit regression line in larvae of *Culex quinquefasciatus* in the laboratory after 72h.

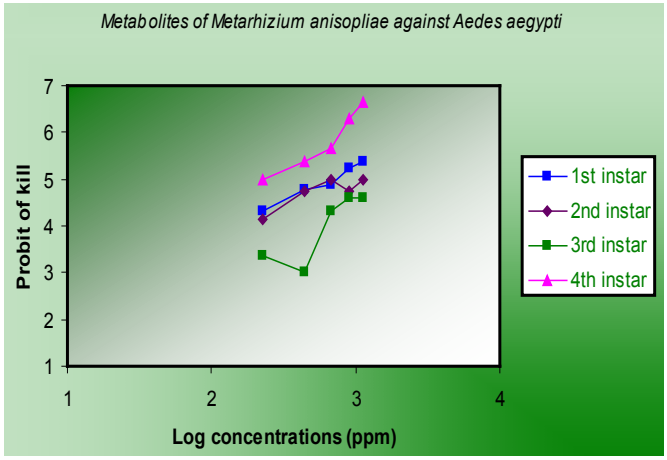

Figure 2: Comparative relationship between probit of kill and log concentrations of *M. anisopliae* filtrate metabolites showing probit regression line in larvae of *Aedes aegypti*, in the laboratory after 72h.

indicate that it was most tolerant to *M. anisopliae* metabolites. A strong positive correlation was found between concentrations of fungal filtrate and percentage mortality of *Cx. quinquefasciatus* larvae (r=0.83, 0.87, 0.84 and 0.72 for all instars). The observed lethal concentrations have shown degree of susceptibility to fungal metabolites amongst the four larval larval stages of *Cx. quinquefasciatus* in order of instar $1^{st} > 2^{nd} > 4^{th} > 3^{rd}$.

The calculated chi-square values at 3 df were 6.22, 1.64, 2.79 and 2.98 for all the instars. The calculated chi square values for *Cx. quinquefasciatus* were lower than critical values of chi-square at 0.05 significant levels. Therefore, the values for chi-square test were statistically not significant at 95% confidence level. It would suggest that there was no significant difference between expected and observed data. The small values of chi-square confirmed the adequate representation of probit regression lines (Figure 1) for the experimental data.

LC values and chi-square analysis for *Ae. aegypti* larvae

Lethal concentration of metabolites are shown in Table 2 with their fiducial limits and probit equations. The probits were plotted against respective log concentration for each instar (Figure 2). LC_{90} value was lower for the third instar than for the other instars. Whereas, the highest value of LC99 was recorderd in first instar . A strong corelation was found between concentrations of metabolites and percentage of larval mortality (r=0.96,0.76.0.69 and 0.90 for the all instars). Lethal concentrations have shown the degree of susceptibility to fungal metabolites among the four instar of *Ae. aegypti* in order of instar $1^{st} > 4^{th} > 2^{nd} > 3^{rd}$.

Chi-square values at 3 df were 0.32, 10.43, 7.09 and for the values of the all instars. The values of Chi-square for 2^{nd} and 4^{th} instars were higher than the original value of chi-square at 0.05 significant only for 2^{nd} and 4^{th} instars at 95% confidential level, which suggest that there was no significant differences between expected and observed data. The small value of chi-square confirmed the adequate representation probit regression line (Figure 2).

Discussion

Mosquito transmitted disease remains a major source of illness. To manage mosquito population, biological control is the use of natural enemies. Microbial larvicides are especially valuable because

Instars	Probit Equations	LC_{50}	95٪ CL	LC_{90}	95٪ CL	LC_{99}	95٪ CL
First Instar	1.2656x + 1.877	97.49	102.91–92.08	243.22	248.63–257.80	663.74	668.91– 658.32
Second Instar	1.5328x + 0.9143	33.11	51.52–14.70	104.77	123.18 – 86.36	466.65	485.06 – 448.24
Third Instar	1.4304x + 1.2455	40.73	45.17 – 36.28	141.25	145.69–136.80	316.66	320.66 –311.77
Fourth Instar	1.5699x + 0.6177	41.68	32.722–50.63	131.82	140.77–122.86	416.86	425.81– 407.90

Table 1: Probit equations and susceptibilities of *Culex quinquefasciatus* against extracellular metabolites of *Metarhizium anisopliae* with 95٪ confidential limit (CL).

Instars	Probit Equations	LC_{50}	95٪ CL	LC_{90}	95٪ CL	LC_{99}	95٪ CL
First Instar	1.495x+0.773	23.98	25.36-22.60	77.62	79.51-75.73	525.80	527.52-522.55
Second Instar	1.100x+1.680	3.09	3.57-2.60	18.19	19.46-16.93	323.59	326.09-321.08
Third Instar	2.265x – 2.285	29.51	32.00-27.01	72.44	74.3-70.58	309.02	310.49-307.55
Fourth Instar	2.294x – 0.552	133.35	133.90-132.79	323.59	323.81-323.36	359.59	359.87-359.36

Table 2: Probit equations and susceptibilities of *Aedes aegypti* against extracellular metabolites of *Metarhizium anisopliae* with 9٪ confidential limit (CL).

their toxicity to non-target animals and human is extremely low. An important benefit of microbial metabolites is that they can be used to replace, at least in part, some more hazardous chemical pest control agents. A number of entomopathogenic fungi have been used effectively to control the mosquito vectors for last few decades. However, studies on the effects of extracellular metabolites on mosquito larvae and non-target organisms appear to be very limited in comparison to the use of spores and mycelia of the fungi [5].

In the present investigation extracellular secondary metabolites of *M. anisopliae* were found effective against the all larvae of *Ae. aegypti* and *Cx. quinquefasciatus*. In both cases the first instars were found more susteptible than third instars. However, among the two vectors *Cx. quinquefasciatus* was found more susceptible than *Ae. aegypti*. Auther has also used the same concentration of this metabolites against the *An. stephensi* and non target aquatic organisms in the laboratory and According to this studies, metabolites of *M. anisopleai* was found safe for the nontargets [5]. Our present investigation of the efficacy can be further extended with the test of isolated molecules on mosquito larvae. In other studies, *Lagenidim giganteum* [8-10], *Tricophytom ajjeloi* [11], *Chrysosporium tropicum* [12] were also tested in the laboratory against mosquito larvae. *Beauveria bassiana*, *Paecilocyces fumosoroseus*, and *Fusarium moniliforme* produce mosquito larvicidal compound like cyclodepsipeptide, including beauvericin and the enniatin complex [13].

Fungal metabolites have the greatest potential in intelligently designed and carefully applied in mosquito management programes. Expanded use of microbial larvicide will depend heavily on the balance between production costs and ecological considerations. Fungal metabolites could be alternative source for mosquito larvicides because they constitute a potential source of bioactive compounds and generally free from harmful effects. Use of fungal metabolites in mosquito control instead of synthetic insecticides could reduce the cost and environmental pollution. Further studies on identification of active compounds, toxicity and field trials are needed to recommend the active fraction of microbial metabolites for development of eco-friendly stategies for the control of the mosquito vectors.

Acknowledgments

Author is grateful to the Director of the Dayalbagh Educational Institute for providing experimental facilities. The financial support was provided by Department of Science and Technology to Prof. Soam Prakash is also acknowledged.

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