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Development of fungal entomopathogen *Beauveria bassiana* (Balsamo) formulations for control of mosquito larvae in the field

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ABSTRACT

Till date, malaria remains a major global problem, exacting an unacceptable toll on the health and economic welfare of the world's poorest communities. To overcome the problem, application of chemical insecticides, has been in use for decades, but it has met with tremendous setbacks in the light of the development of vector resistance and some attendant environmental hazards.In our study, we have focused on an ecofriendly entomopathogenic fungus to know its efficacy to control mosquito larvae in field trial. To evaluate the efficacy of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) on malaria causing mosquitoes in the field, outdoor trials were undertaken in Udaipur city. The entomopathogenic fungus *Beauveria bassiana* (Balsamo) have demonstrated effectiveness against anopheline larvae in the laboratory. Laboratory reared *Anopheles stephensi* (L.) second instar larvae were selected for field studies. The powdered form of fungus mixed with CMC (Carboxy Methyl Cellulose) (5 g/kg) were obtained. In the field bioassay, doses i.e., 1 g, 2.5 g and 5 g of fungal conidia were tested. The data clearly revealed the effectiveness of *Beauveria bassiana* (Balsamo) in field conditions. The study demonstrated that the entomopathogenic fungus *Beauveria bassiana* (Balsamo) caused high impact on the survival of *Anopheles stephensi* (L.) in outdoor trials, when formulated in CMC (Carboxy Methyl Cellulose).

Key words : *Beauveria bassiana, Formulations, Mosquito, Larvae, Control, Field*

Introduction

A deadly disease Malaria is caused by parasites that are transmitted to individuals through the mosquitoes bites of infected female Anopheles. There were an estimated 229 million malaria cases globally in 2019. In 2019 the estimated number of malaria deaths stood at 4,09,000. Under 5 years'children aged are the most susceptible group affected by malaria. The WHO African Region carries an excessively high share of the global malaria problem (WHO, 2021). Firstly, a large number of cases are not reported every year, so it is difficult making precise estimations. Secondly, the world's population is growing, and it is growing at the greatest rate in Africa, where the majority of malaria cases occur. Even if the percentage of people with malaria decreases over time, due to health initiatives such as distributing long-lasting insecticide treated bed nets, free treatment etc. the total number of cases may still increase. Another problem in the combat against malaria is climate change. World's patterns of temperature and rainfall are changing, new areas become vulnerable to malaria transmission, putting

more people at threat. Apart from children, pregnant women are also very susceptible to malaria infection. Malaria in pregnancy is a major public health problem in endemic countries. It is recurrently reported that primigravidae and secundigravidae are the most at risk (Brabin, 1991), and much of the works on the problem of malaria during pregnancy has focused on women of these gravidities (Steketee *et al.*, 1996). Approximately 25 million pregnant women are at risk of *Plasmodium falciparum* infection every year in Sub-Saharan Africa. *Plasmodium falciparum* infections can cause severe maternal anemia, low birth weight and prenatal mortality. Low birth weight associated with malaria during pregnancy is estimated to result in 100,000 infant deaths in Africa every year (Desai *et al.*, 2007).

In India, currently 80.5 percent of the population of India lives in malaria risk zones. Of this, 4.2 percent, 32.5 percent and 43.8 percent live in areas of high, moderate and low risk to malaria respectively (Dash *et al.*, 2008). At present NVBDCP, (National Vector Borne Diseases Control Programme) indicate 1.5–2 million confirmed cases and about 1,000 deaths annually (Kumar *et al.*, 2007). During 2009, total 2.7 million confirmed malaria cases (Microscopically and Rapid Diagnostic Test (RDT)) and 3188 malaria deaths were reported in the Southeast Asian Region where as estimated malaria cases were around 26 -36 million and malaria deaths between 42300 – 77300. The *Plasmodium falciparum* proportion remained around 60.5 percent (including RDT positives). Of these, the highest number (1,563,344) of laboratories confirmed cases were reported from India.

According to World malaria report 2019, an estimated 228 million cases of malaria in 89 countries and in 2018 malaria deaths were 405000. Most malaria cases in 2018 were in African Region (93%), trailed by South-East Asia Region with 3.4% of the cases and Eastern Mediterranean Region with 2.1%. South-East Asia Region, three countries India (58%), Indonesia (30%) and Myanmar (10%) total for 98% of the total reported cases in the region. India represents 3% of the worldwide malaria problem (WMR, 2019).

Occurrence of insecticide resistance in mosquitoes and other public health pests have been reported (Waseem *et al.*, 2011). Their residues in the environment and effects on humans and nontarget organism are major problems.

The goal of mosquito control in malaria should be, to control mosquitoes in safe, efficient and cost-effective manner and while doing so, prevent damage to humans, animals, land and natural environment. This task can be successfully accomplished through the integrated vector control approach known as Integrated Vector Management.

In our study, a field experiment was conducted to evaluate the efficacy of *Beauveria bassiana* (Balsamo), an entamopathogenic fungus on larvae of malaria causing mosquitoes.

Materials and Methods

To evaluate the efficacy of *Beauveria bassiana* (Balsamo) in the field, experiment was conducted in area of Udaipur. Malaria is prevalent in this region and transmission occurs throughout the year. Field study and toxicity of fungus were done according to Bukhari *et al.*, 2011.

Outdoor field bioassay was conducted in 15 plastic containers (15.5cm in diameter) (Figure 1). Dry soil from field was softened up by adding water. The softened soil was placed at the bottom of each plastic container to form a 2 cm thick layer. 500 ml of tap water was then added to each plastic container. The water level was 3 cm above soil level. Each plastic container was placed in a larger tray that also was filled with water to the top. The larger trays were employed to prevent ants from accessing the plastic container inside (Figure 2). Fifty second-instar *Anopheles stephensi* (L.) larvae, were added to each container. The large trays, with the containers inside, were arranged in three rows some distance apart from each other.

Sets of three replicas of plastic containers containing 50 second instar larvae of *Anopheles stephensi* (L.) each were taken for the study. In set one, different selected doses *i.e.*,1g, 2.5g and 5g of 1×10^8 spores/g of *Beauveria bassiana* (Balsamo) were applied in water. The fungus was mixed with CMC (Carboxy Methyl Cellulose) for sticking of fungal spores (for field studies, dry formulation of *Beauveria bassiana* (Balsamo) were used).

Another three plastic containers were provided with 50 second instar larvae and 0.025 g, 0.0125 g and 0.005 g of CMC (these doses were calculated according to the amount of CMC mixed with fungal preparations) to evaluate the effect of CMC on larvae (Since dry preparation of *Beauveria bassiana* (Balsamo) obtained contained 5 g CMC/Kg of fungal spore powder.)

Fig. 1. Field experiment protocol
Fig A &B. 50 No. of second instar larvae placed in each plastic containers containing *B. bassiana* fungus, CMC and control treatment with 2 cm soil layer and water 3 cm.
Fig. C. Later Small containers were covered by lids to see adult emergence

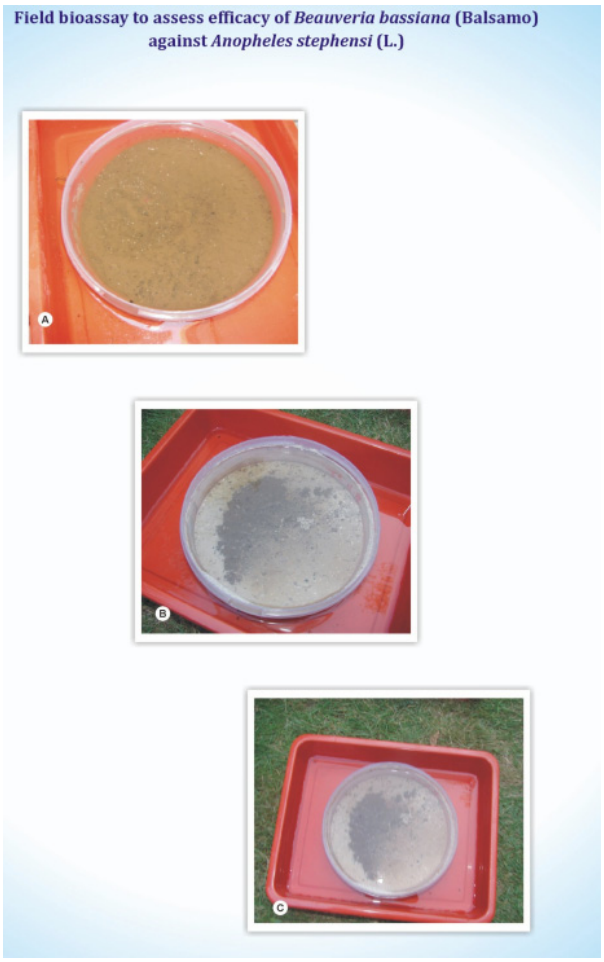

Fig. 2. Field bioassay to assess efficacy of *B. bassiana* against *Anapheles stephensi* (L.)
Fig A. Plastic containers without fungal dose i.e. control.
Fig B & C. Formulated *B. bassiana* fungus conidia applied on the water surface.

Third set contained only 500 ml of water with 50 second instar larvae to serve as control.

Results

When different doses, i.e. 1,2.5 and 5 g of fungal powder with a concentration of 1×10^8 conidia/g were mixed in 500 ml of water and 50 second instar larvae of *Anopheles stephensi* (L.) were kept till pupation (as per day larval mortality was not possible to observe due to sand on the bottom of container), 12.67, 5.67 and 0.33 pupae emerged at 1, 2.5 and 5.0 g of 1×10^8 conidia/g after thirteen day respectively as compared to 37.00 and 35.00 mean of larvae in CMC and control sets respectively (Table 1a).

The ANOVA revealed highly significant difference in number of pupae emerged in different concentrations. For further analysis, Tukey's Post Hoc test was applied and the results were interesting. The Post Hoc test revealed that control and CMC group and, 2.5 and 5 g (group) were not significant thus both formed a homogeneous group. Further significant difference was observed in control and all applied doses i.e., 1, 2.5 and 5.0 g of 1×10^8 conidia/g and CMC and *Beauveria bassiana* (Balsamo) doses Table 1(b). Here it was also indicated that CMC is not exerting any lethal effect on *Beauveria bassiana* (Balsamo) spores. The Tukey's test also revealed significant difference between 1, 2.5 and 5.0 g of 1×10^8 conidia/g.

Table 1. Field study on *Beauveria bassiana* (Balsamo) against Anopheles mosquito fauna.
(a): Number of Pupa Emerged

Category	N	Mean	SD	F	Df	Result
Control	3	35.00	3.00	138.22	4.10	***
CMC	3	37.00	4.00			
1 g	3	12.67	2.08			
2.5 g	3	5.67	1.15			
5 g	3	0.33	0.58			

(b): POST-HOC – TUKEY'S TEST

	Control	CMC	1 g	2.5 g	5 g
Control	-				
CMC	NS	-			
1 g	*	*	-		
2.5 g	*	*	*	-	-
5 g	*	*	*	NS	-

Discussion

The entomopathogenic fungus *Beauveria bassiana* (Balsamo) have demonstrated effectiveness against anopheline larvae in the laboratory. However, utilizing the fungi for the control of anopheline larvae under field conditions relies on development of effective means of application as well as reducing their sensitivity to U.V. radiation, high temperatures and the inevitable contact with water.

To evaluate the efficacy of the entomopathogenic fungus *Beauveri abassiana* (Balsamo) in the field, outdoor trials were undertaken in Udaipur city. Recently, theoretical and experimental studies have shown the potential of entomopathogenic fungi as next-generation agents for the control of malaria mosquitoes (Hancock, 2009; Hancock *et al.,* 2008 and Knols *et al.,* 2010). However, most of the work has focused on targeting adult mosquitoes but larval control has a convincing history of malaria eradication and recent studies have also shown this approach to be highly effective (Killeen *et al.,* 2002a, Killeen *et al.,* 2002b and Fillinger, 2009a). It is therefore, worthwhile to investigate the ability of entomopathogenic fungi to control mosquito larvae and the feasibility of their operational use.

Laboratory reared *Anopheles stephensi* (L.) second instar larvae were selected for the field studies. Pathogenicity of biological control agents in the field is generally lower than that in the laboratory settings (Becker and Rettich, 1994). So, in the field bioassay, higher doses, i.e. 1 g, 2.5 g and 5 g of fungal conidia were tested.

When 1g, 1.5 g and 2.5 g×1×10^8 conidia/g/kg were tested against second instar larvae in field conditions (dry formulations), the percent pupal emergence was at par in control (35.00) and CMC (Carboxy Methyl Cellulose) (37.00) which is, used as adjuvant, whereas the fungal doses of 1 g, 1. 5 g and 2.5 g ×1×10^8 conidia/g/kg after thirteen days brought about 12.67, 5.67 and 0.33 percent pupal emergence respectively. The 'F' value was significant (138.22) at 4.10 degree of freedom. The data clearly revealed the effectiveness of *Beauveria bassiana* (Balsamo) in field conditions.

Gutiérrez and Luna, (2008) have found that the virulence of strains BbZ3 and BbZ4 of the entomopathogenic fungus *Beauveria bassiana* was determined by introducing infested cadavers of *Galleria mellonella* L. through the orifices on the stem pads of the nopal plant. Both stains of *Beauveria bassiana* were highly pathogenic causing 100 percent mortality in the larvae of *Laniifera cyclades* inside the nopal cladodes in the greenhouse as well in the field. BbZ3 was more virulent with a LT_{50} of 5.1 days in the greenhouse and 6.4 days in the field, while the LT_{50} of BbZ4 was 6 and 7.5 days, respectively. The application of larval cadavers of *G. mellonella* infested with the fungus *B. bassiana* was an effective control strategy against larvae of *Laniifera cyclades.*

Since fungal preparation was formulated in powder form by using CMC (Carboxy Methyl Cellulose) powder. As dry preparation has been reported to be effective against mosquito larvae. According to Alves, (2002), most of the studies were carried out in the laboratory and proved the application of dry

fungal conidia to be more effective than the application of formulated conidia.

Formulations can have a considerable impact on improving the efficacy of bio pesticides. An ideal formulation aids the handling and application of the bio pesticides, as well as increases the efficacy by improving contact with the host and protecting the active agent from environmental factors (Goettel, 2005). Considering the surface feeding behavior of anopheline larvae, any formulation intended to infect them should spread the fungal conidia over the water surface (Merritt, 1992). The conidia should spread uniformly, providing equal coverage, over the entire treated area. In addition, conidia should be prevented from germinating before host attachment, and at least to some extent be protected from environmental factors. Simultaneously, it should not be toxic for the fungus. The at par result of control (untreated) and CMC (35.00 and 37.00) further supported the use of CMC as good binder for dry formulation of fungus. Further, the Tukey's Post HOC test results showed non – significant difference between CMC and control group, and 2.5g and 5g, as mean number of pupae emerged in CMC and control group was 37.00 and 35.00, whereas in 2.5g and 5g was 5.67 and 0.33.

It was observed in other studies that under field conditions the conidia are more likely to lose their pathogenicity quickly due to exposure to high temperatures and UV radiation. Hence, results in field showed, how formulations can improve the ability of entomopathogenic fungus conidia to spread over a water surface as well as increase their persistence. The study demonstrated that the entomopathogenic fungus *Beauveria bassiana* (Balsamo) caused high impact on the survival of *Anopheles stephensi* (L.) in outdoor trials, when formulated in CMC (Carboxy Methyl Cellulose).

Carboxy Methyl Cellulose (CMC) can be considered as a candidate carrier that not only facilitated the application of conidia but also improved their efficacy by providing maximum chance for contact (spreading the conidia on the water surface) with the larvae and increasing conidium persistence. The fungal conidia readily suspend in CMC with a slight agitation. So, the conidia can be conveniently mixed in CMC on spot, which means that during transportation and storage only the bio – active agent would have to be kept at low temperatures rather than the whole mixture. This can reduce the cooling space requirements as CMC itself is a stable product and has no particular storage demands. In our experiment, when only CMC was used at same dose level of 1.0, 2.5 and 5 g per replicate of 500 ml of water, the Percent pupal mortality was nearly same as that of control that proved CMC to be a low toxicity carrier.

The mosquito controlling effects of the fungus not only reduced larval density, but also stunted their development. The fourth instar occupied a smaller part than that in control group, and the proportion of pupal stages was almost less than that of control. This suggests that under the effects of the fungus, larvae were sick or developed abnormally, and only a small number of them could pupate. Some pupae were observed dying from mycosis. As a result, the adult number was even smaller.

Further, lower and higher doses revealed same mortality level (2.5 and 5 g), showing that 2.5 g and 5 g of fungal dose gave non-significant difference between them (Tukey Post -Hoc test). So, it can be considered that for field experiments higher doses can be replaced by lower doses for controlling anopheline larval population.

Conclusion

Thus, the finding of new mosquito – killing microbe is significant to mosquito integrated pest management for some reasons, firstly, it helps to extend the range of applicable agents to meet the needs of mosquito bio control under different ecological conditions. Secondly, native agents could perform better and thirdly, knowledge of this fungus may help enrich the pool of mosquito - killing genes for the purpose of developing more effective and ideal agents for controlling mosquitoes using genetic engineering techniques. Hence to sum up source reduction can be one of the important determining factors in malaria control strategy. Many breeding sites of vector are man-made and it does not make sense to keep those aquatic habitats for producing malaria vector. WHO (2004) also emphasized on source reduction and said that 'The main impact of environment management (including source reduction) is to prevent or minimize propagation (WHO 2004).

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