Ashdin Publishing
Malaria Chemotherapy, Control & Elimination
Vol. 1 (2012), Article ID 235494, 6 pages
doi:10.4303/mcce/235494

Research Article

Evaluation of Entomopathogenic Fungus *Metarhizium anisopliae* Formulated with Suneem (Neem Oil) against *Anopheles gambiae* s.l. and *Culex quinquefasciatus* Adults

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Received 22 August 2011; Revised 16 March 2012; Accepted 4 April 2012

Abstract Biological control using pathogenic fungi is a promising alternative to chemical control. In this study, the pathogenicity of *Metarhizium anisopliae* formulated with neem oil (Suneem 1%) was evaluated against *Anopheles gambiae* s.l. and *Culex quinquefasciatus* adults. Under laboratory conditions, conidia were sprayed into $30 \times 30 \times 30$ cm netting cages at 6×10^7 spores/ml. With neem oil formulation, the percentages of surviving adults after 4 days were from 67 ± 3.4 to $5 \pm 0.5\%$ for *An. gambiae*, and from 51 ± 4.1 to $12 \pm 1.1\%$ for *Cx quinquefasciatus*. With the aqueous formulation, the survival rates were from 97 ± 3.2 to $58 \pm 2.1\%$ and 95 ± 2.5 to $70 \pm 2.1\%$ for *An. gambiae* and *Cx quinquefasciatus*. Very low mortality was observed in the water control. *M. anisopliae* in Suneem formulation could be developed for a spray technique, before being introduced in vector control.

Keywords *M. anisopliae*; neem oil; entomopathogenic fungi; *An. gambiae*, *Cx quinquefasciatus*; biological control

1 Introduction

Mosquitoes such as *Anopheles gambiae* and *Culex quinquefasciatus* are responsible for the transmission of several parasites that cause diseases such as malaria and filariasis. In Senegal, the high infection rates of malaria are mainly due to rapid urbanization [12,25,32,35,45]. Chemical and mechanical methods are the most common for mosquito control. One of the major strategies in malaria elimination is protection using insecticide-treated nets [24,44], and more recently, long lasting insecticidal nets (LLINs).

In mosquito vector control, many efforts have been made in developing countries using insecticides. However, the continued use of this method has resulted in the development of mosquito resistance [1,6,48]. For an alternative to chemical control, there is a resurgence of interest in the use of biopesticides. Therefore, biological control is an important component of the integrated vector control strategy. Among various biocontrol agents, plant extracts [8,31,34,42], bacteria [14] and entomopathogenic fungi [13,23,29,33,38,39,40] belong to the most promising groups used for mosquito control. These agents are being used in many countries for insect control. Although, in Senegal, mosquito biological control is less used.

Many studies have showed the effectiveness of entomopathogenic fungi for mosquito control [23,29,33,38,39]. These fungi infect mosquitoes through direct contact with the cuticle. But, for these agents, there is a problem related to a correct formulation of fungal spores to facilitate spraying against mosquito adults. Studies have shown the possibility of combining fungal spores with plant extracts [40], with chemical insecticides [11,19,50], and also in the form of aggregates [5] against insects. Mahmoud [26] and Mnyone et al. [27] showed the possibility of combining species of entomopathogenic fungi against insects. Recently, oil formulations of entomopathogenic fungi produced satisfactory results in insect control [22] and control of mosquitoes [27, 47]. Some authors, have found low efficacy from the combination of neem oil with fungi [2,7,16], although not all [36, 47]. Among the neem oils, Suneem has not been evaluated for *M. anisopliae* formulation against mosquito adults.

The objective of this study is to demonstrate the possibility of formulating *Metarhizium anisopliae* with neem oil (Suneem) manufactured in Senegal against the adults of *Anopheles gambiae* s.l. and *Culex quinquefasciatus* mosquitoes.

2 Materials and methods

2.1 Sampling of mosquitoes

Larvae of *Anopheles gambiae* s.l. and *Culex quinquefasciatus* were collected from different areas in the suburbs of Dakar: Thiaroye sur mer ($14°44'31''$N and

17°23′53″W), Sam-Sam III (14°45′41″N and 17°21′25″W) Pikine rue 10 (14°45′32″N and 17°23′53″W) Pikine Niety Mbar (14°46′04″N and 17°22′32″W) and Guediawaye (14°46′55″N and 17°22′00″W). Sampling sites included various water bodies: streams, irrigation canals, drainage canals, and temporary water. Larvae were collected and transported in jars containing water from the breeding sites. At the Laboratory of Reproductive Biology (U.C.A.D.), larvae were separated and identified according to [15,18]. After emergence, adults were fed with sucrose solution at 10%. Identification is also made on the adult stage to confirm the identification at the larval stage of the various species.

2.2 Formulation of the fungus

The *Metarhizium anisopliae* is a local strain isolated on *Oedaleus senegalensis* Krauss, 1877 (Orthoptera: acrididae), at the Laboratory of Reproductive Biology, Department of Animal Biology, University Cheikh Anta Diop of Dakar in 2006. The fungus was replicated on rice grains medium in sterilized Petri dishes of dimensions 9 cm in diameter and 3 cm deep. Conidia were aseptically harvested 15 days later and kept in a Pyrex glass bottle sterilized at 110 °C. The sporulation rate was 90%. We used the Suneem 1% for the oil formulation, and distilled water for the aqueous formulation. The Suneem is emulsifiable neem oil formulated with a biodegradable solvent tetrahydrofurfuryl alcohol (THFA). It is obtained from a Senegalese chemical industry (SENCHIM). For oil formulation, 10 mL of Suneem 1% was mixed with 2 g of dry conidia in graduated tube and homogenized for 15 min. After determination of spore content, we diluted with 500 mL of sterile distilled water to obtain a final dose of 6×10^7 spores/mL. The spore content of this solution was determined with a hemacytometer counter (Thoma model) and a magnifying microscope (400×).

The aqueous formulation was prepared according to the same methodology with the same volume of sterile distilled water. The final content after dilution was 6×10^7 spores/mL.

For the oil formulation, preliminary tests showed that Suneem did not inhibit sporulation of the fungus.

2.3 Spraying adults

In each of four $30 \times 30 \times 30$ cm bed net netting cages, were placed 50 males and 50 females (none blood fed). Mosquitoes were 5–7 days old. With a hand sprayer, we applied the product through the mesh of mosquito bed net into the cage to reach the mosquito adults. In each cage one of four treatments was applied: (1) 20 mL of the oil formulation with a dose of 6×10^7 spores/mL (1.3×10^{10} spores/m^2); (2) for the Suneem oil control group, we applied 20mL of a solution of 20 mL Suneem diluted with distilled water to 500 mL; (3) 20 mL of aqueous formulation at 6×10^7 spores/mL (1.3×10^{10} spores/m^2); (4) aqueous control treated with 20 mL of distilled water only. The conditions were 25 ± 1 °C and 75 ± 2% relative humidity (RH).

The dead adults were removed from the cages and placed for incubation on Whatman paper imbibed with distilled water in glass Petri dish previously sterilized. The incubation is done in laboratory conditions (25 °C and 75% RH) for fungal growth. After fungal germination on the cadavers, we visualized sporulation with a magnifiying microscope (×40) with Motic advanced software and connected to a computer.

Adults were fed with 10% sucrose solution during treatment. The mosquitoes were left in the same cages after treatment. The experiment was replicated three times on different days for each test. The results represent the arithmetic means.

2.4 Data recording and analysis

After spraying, we counted the survival of adults daily to calculate the percentage mortality rate. The results represent the average of the three replicates and were used for statistical processing with the Statview software. A paired T-test is also used to verify the sensitivity of both species for the two formulations.

3 Results

After application of *Metarhizium anisopliae* in Suneem oil formulation, or application of Suneem oil control, we found adult mosquito agitation followed by a rapid knockdown. This was not the case for the aqueous formulation or aqueous control application. After a short time (15 min), the mosquitoes recovered, flew and rested on the wall of mosquito nets. 24 hours later, the percentage adult survival among those treated with both formulations decreased (Figures 1 and 2). However, with the Suneem formulation, the survival days of the *Anopheles gambiae* and *Culex quinquefasciatus* adults were lower than Suneem oil control, the water formulation or the water control. For Suneem oil formulation, the percentages of survival adults during the 4 days were from 67 ± 3.4 to 5 ± 0.5% for *An. gambiae*, and from 51 ± 4.1 to 12 ± 1.1% for *Cx quinquefasciatus*. With Suneem oil control the survival of adults at the day 4 were 82 ± 2.5 for *An. gambiae* and 89 ± 1.3 for *Cx quinquefasciatus*. With the aqueous formulation, the survival rates were from 97 ± 3.2 to 58 ± 2.1% for *An. gambiae* and 95 ± 2.5 to 70 ± 2.1% for *Cx quinquefasciatus*. There was low mortality in the water control (< 3%). For both formulations, the paired T-test shows high significant difference in survival rates between treated adults and control for both the oil and the water formulations ($p < 0.0001$) (Tables 1 and 2). Furthermore, univariate T-test shows that the oil formulation is more effective against *Anopheles gambiae* ($p = 0,001$) and *Culex quinquefasciatus* adults ($p = 0,002$) than water formulation.

Table 1: Percentage survival of adults (mean ± SE) of *Anopheles gambiae* s.l. sprayed with *Metarhizium anisopliae* in neem oil (Suneem) and water formulation at 1.3×10^{10} spores/m^2.

Days post application	Suneem oil formulation	Suneem oil control	p value	Water formulation	Water control	p value
	Average percentage survival ± SE			Average percentage survival ± SE		
D1	67 ± 3.4	100 ± 0.0	< 0.0001	97 ± 3.2	100 ± 0.1	= 0.0678
D2	61 ± 2.1	93 ± 1.4	< 0.0001	94 ± 2.4	99 ± 1.8	= 0.0008
D3	14 ± 1.5	86 ± 2.1	< 0.0001	82 ± 1.5	99 ± 4.2	< 0.0001
D4	5 ± 0.5	82 ± 2.5	< 0.0001	58 ± 2.1	98 ± 2.1	< 0.0001

Table 2: Percentage survival of adults (mean ± SE) of *Culex quinquefasciatus* sprayed with *Metarhizium anisopliae* in neem oil (Suneem) and water formulation at 1.3×10^{10} spores/m^2.

Days post application	Suneem oil formulation	Suneem oil control	p value	Water formulation	Water control	p value
	Average percentage survival ± SE			Average percentage survival ± SE		
D1	65 ± 4.1	100 ± 0.0	< 0.0001	95 ± 2.5	100 ± 0.0	= 0.0008
D2	58 ± 2.2	96 ± 1.2	< 0.0001	84 ± 1.8	98 ± 0.2	< 0.0001
D3	48 ± 2.0	95 ± 2.1	< 0.0001	75 ± 4.2	98 ± 0.1	< 0.0001
D4	12 ± 1.1	89 ± 1.3	< 0.0001	70 ± 2.1	97 ± 0.0	< 0.0001

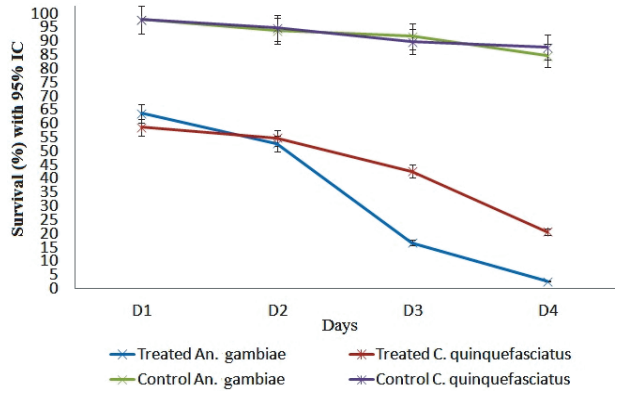

Figure 1: Effect of *Metarhizium anisopliae* in Suneem (neem oil) formulation on *Anopheles gambiae* and *Culex quinquefasciatus* survival. For the control, the mosquitoes are treated with Suneem.

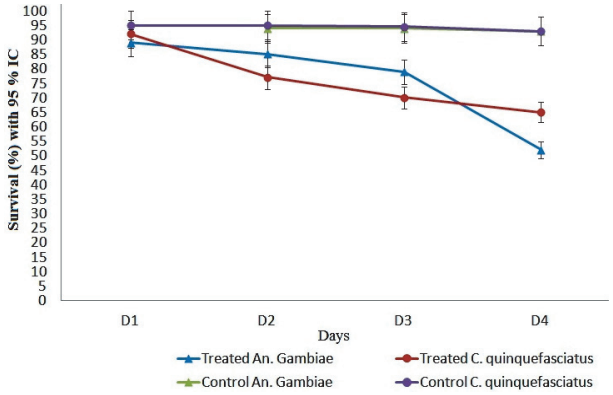

Figure 2: Effect of *Metarhizium anisopliae* in water formulation on *Anopheles gambiae* s.l. and *Culex quinquefasciatus* survival. For the control, the mosquitoes are not treated with fungus.

After 7 days incubation on Whatman paper in glass Petri dish previously sterilized, we observed the germination of the fungus on all adult mosquitoes treated with conidia and incubated (Figures 3(a) and 3(b)). For both formulations, germination showed no difference in sporulation of the fungus on *An. gambiae* and *Cx quinquefasciatus*. This germination was observed on the head, thorax, and abdomen of adult mosquitoes. However, no germination was observed on either of the control groups (Figures 3(c) and 3(d)).

4 Discussion

In this study, the pathogenicity of *Metarhizium anisopliae* formulated with neem oil (Suneem) has been demonstrated against adult mosquitoes of *Anopheles gambiae* and *Culex quinquefasciatus*. When formulation was sprayed on mosquitoes, their survival was significantly reduced. This supports previous laboratory trials that have demonstrated the potential of *Metarhizium anisopliae* for adult mosquito control [37,38,39]. In our bioassays, the Suneem 1% showed no inhibitory effect on spore germination or reduction in spore pathogenicity to the treated mosquitoes. Instead, the results showed that the oil formulation is more effective against mosquitoes than the aqueous formulation or Suneem alone. That confirms our previous results in synergism effect between Suneem and entomopathogenic fungi (*Aspergillus clavatus*) at 79×10^7 spores/mL against adult of *Culex quinquefasciatus* [41]. Indeed, many studies have shown the possibility of combining neem oil with entomopathogenic fungi for insect control [21,30]. However, some oils are not very compatible for conidial formulation [7,16,36]. Therefore, the oil facilitates not only the spraying fungal spores [4], but it plays a role of synergism [7,41] and facilitates their adhesion to the insect cuticle [46,47]. This is a great advantage to the

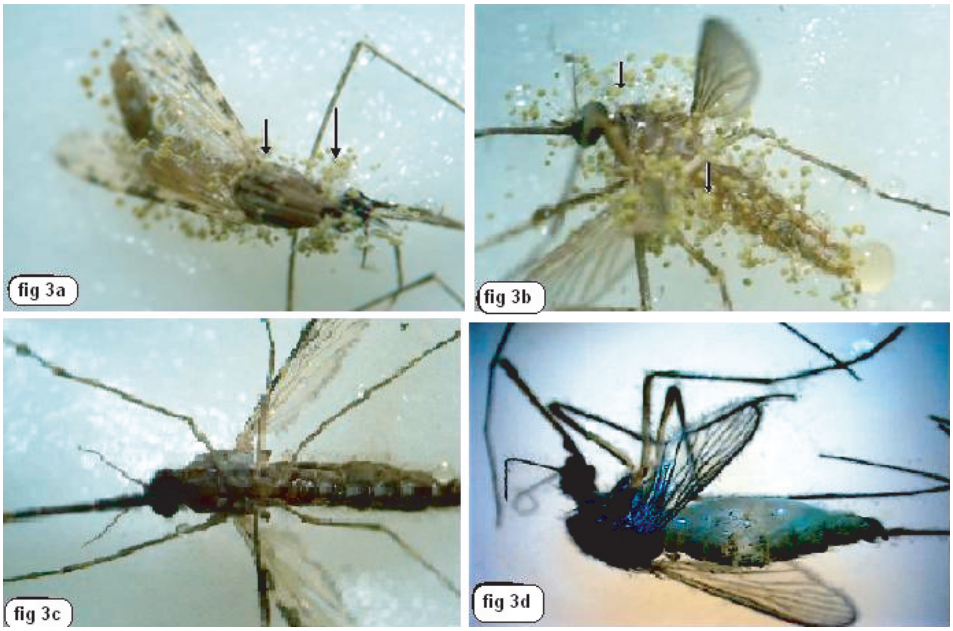

Figure 3: *Anopheles gambiae* (a) and *Culex quinquefasciatus* (b) mosquito adults infected by *Metarhizium anisopliae* and none infected (control) (c,d).

mix. The contribution of Suneem is also the beneficial effect as biopesticide, which was demonstrated on mosquito larvae [42]. But the Suneem, used in this study, was more diluted. That is why it has not been so pathogenic to the mosquito adults compared to other studies [9, 10].

On the other hand, the choice of the oil is therefore essential to increase the effectiveness of the fungus. Some oils can effectively inhibit the germination of spores, thus affecting their effectiveness [19]. So, the composition or origin of neem oil [43], used in the formulation is most important for conidial effectiveness.

In our bioassays, mosquito behavior observed immediately after spraying the oil formulation and Suneem alone, shows agitation and excito-repellency effects, as is previously reported [49], also in addition to a knockdown effect. However, in our study, this effect is ephemeral and could be due to the volatile solvent present in the Suneem (THFA) or the oil dilution with water.

After spraying, the conidia need to contact the mosquito adult, after which they attach, germinate, and penetrate the cuticle. The Suneem, which is an emulsifiable oil plays a facilitating role for the adhesion of spores on the cuticle of insects.

In this study, the dose used against *An. gambiae* and *Cx quinquefasciatus* was 6×10^7 spores/mL, which is lower than the dose used by Kannan et al. [23] against *Anopheles stephensi* (1×10^8 spores/mL of water or oil suspension), or *M. anisopliae* formulated with sunflower oil against the same mosquito species at 1.6×10^{10} spores/mL [39]. However, the mode of application is not the same. Our formulation was sprayed directly on the mosquitoes but conidia were also attached in the bed net upon which mosquitoes rested. Mosquitoes were then continuously in contact during 4 days with bed net. This also shows that the application method influences the effectiveness of the product. Even if, for Farenhorst and Knols [10], the use of a standardized application method (on substrates), allows optimizations of spore dose and exposure time. In our study, we sprayed directly through bed net on mosquitoes, while the modes of application for regular laboratory tests often use paper filters [14, 38] or other substrates [10, 20, 28]. But we can explain also the higher mortality in our study, by the difference of the strain used against the same mosquitoes. The content of the entomopathogenic fungi formulation (strain, conidial dose, nature of neem oil) is important, but also the nature of the treated surface and contact areas of mosquitoes with spores (tarsus, head, thorax, abdomen, antennae) as is body size. Mnyone et al. [28] shows that older and non-blood fed mosquitoes are more susceptible than younger or blood fed. The mosquito adults used in our study were between 5 and 7 days old and not blood fed

Since the germination of fungal spores depends on the humidity and some factors [17], and the most vital parts of the body (head, thorax, and abdomen) showed more germination of conidia, contact with the tarsi or antennae would be less infectious. So that, if the application of a fungus is through substrate only [20], adult mosquitoes will be in contact by their legs or antennae, and rarely by the rest of the body. Then, the infection rate is not high during the first day and the lethal time will increase.

In the other hand, there are possibilities of fungal dissemination amongst the mosquito adults [37]. We used 50 males and 50 females in the same cage during 4 days. The infection may spread among the mosquitoes by bodily contact. Then, the number of mosquito adults male and female treated is also important due to activities as mating within mosquito population. If more conidia are adhered to the mosquito adult cuticle, the possibility to infect another by contact must be enhanced. Furthermore, fungal infections suppress the successful development of Plasmodium parasites in the vectors [3], which should be investigated in this location.

5 Conclusion

Metarhizium anisopliae strain used in our study is compatible with Suneem 1% and reduces survival of mosquito adults after spraying. The mosquito age and time of contact between adults and conidia must enhance the mosquito infection. The possibility exists to use entomopathogenic fungi formulated with Suneem against mosquito adults as malaria mosquito vector control in Senegal. Therefore, a technical spray similar to that described here should be developed for use in the field environment to target host-seeking or house entering mosquitoes.

Acknowledgments The authors thank Dr Toure for the *Metarhizium anisopliae* strains and Dr Ndione for mosquito sampling. This study was supported by the Ministry of Scientific Research of Senegal in the project program "FIRST" 2008.

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