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Laboratory and semi-field environment tests for the control efficacy of *Metarhizium anisopliae* formulated with neem oil (suneem) against *Anopheles gambiae* s.l. adult emergence

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ABSTRACT

Metarhizium anisopliae was evaluated previously in Suneem formulation against malaria vector adults. However, their ability to control aquatic stages is not yet evaluated. In laboratory conditions: the lethal dose (LD_{90}) of the formulation was determined on *Anopheles gambiae* larvae collected from breeding sites and evaluated into artificial vats at dry and rainy seasons. In laboratory conditions, the LD_{90} was 5.3×10^6 spores/ml in 48 h. In semi-field environment, the formulation had a great emergence inhibition of mosquito adult ($P < 0.0001$). The emergences rate at day 8 were 2.25 ± 0.03, 28.00 ± 1.07 and 97.25 ± 1.56 % in dry season for the oil formulation (OF), Suneem (S), and water control respectively. In rainy season, the emergences were 1.25 ± 0.15, 30.25 ± 1.23 and 98 ± 0.76 % respectively. No significant difference was observed between dry and rainy seasons ($P=0.3$). Therefore, *M. anisopliae* formulated with Suneem may provide a more sustainable management strategy for malaria vectors control at larval stages.

Key words: *Metarhizium anisopliae*, *Anopheles gambiae* s.l., neem oil, biological control.

INTRODUCTION

In Senegal, the high infection rates of malaria are mainly due to rapid urbanization (Fontenille et al., 1997**;** Robert et al., 2006; Pagès et al., 2008). With significant rainfall recorded since 2005, some suburbs areas of Dakar are frequently flooded due to the shallow groundwater in the Niayes zone, this result in the development of anopheline mosquitoes during rainy and dry season (unpublished data).

Currently, many efforts have been made in malaria control by managing the mosquitoes using chemical insecticides. One of the major strategies in malaria elimination in Senegal is the personal protection using Long Lasting Insecticidal Nets (LLINs) (Talani et al., 2005; Kweka et al., 2008; Adeogun et al., 2012). However, the continuous use of chemical insecticides against mosquitoes has caused enormous problems like development of resistance (Akogbeto and Yakoubou, 1999; Weill et al., 2003). Development of alternative methods as biological control using pathogenic agents such as bacteria and entomopathogenic fungi is in necessity.

Many studies have shown the potential of entomopathogenic fungi as next generation agents for the control of mosquitoes (Scholte et al., 2003; 2004; 2006; Kannan et al., 2008; Seye et al., 2009; Bilal et al., 2012; Seye et al., 2012). Among these fungi, *Metarhizium anisopliae* and *Beauveria bassiana* strains are the most used. However, the fungal spores are hydrophobic. When they are applied in aquatic environment, they clump together, reducing the area of contact with mosquito larvae. A formulation is then needed to facilitate the spraying and effectiveness. Many oils from plants have showed compatibility with entomopathogenic fungi (Visalakshy et al., 2006; Seye and

Ndiaye, 2008; Sahayaraj et al., 2011). The combination increases entomopathogens infectivity against the target pest by enhancing conidial adhesion and persistence. However, *M. anisopliae* are more commonly used in oil formulation against mosquito adults (Mnyone et al., 2011; Seye et al., 2012), than mosquito larvae (Bukhari et al., 2001). These authors showed that, the percentage of pupation for *Anopheles gambiae* s.l. was significantly reduced when *M. anisopliae* was used in Shell oil formulation in laboratory and field condition.

Neem oils were also used for entomopathogenic fungi formulation. However, some oils are less compatible with fungi and can inhibit their effect (Bajan et al., 1998; Hirose et al., 2001; Depieri et al., 2005). Suneem, an emulsionable neem oil manufactured in Senegal, was formulated with *M. anisopliae* against *A. gambiae* adults (Seye et al., 2012). However, the effectiveness of this formulation against larvae in aquatic medium was not investigated. It is therefore, worthwhile to evaluate *M. anisopliae* in emulsible Suneem formulation against *A. gambiae* larvae as potential application method for mosquito control in the field.

The aims of this study is to determine the LD_{90} of *M. anisopliae* formulated with Suneem on *A. gambiae* larvae in laboratory conditions and to evaluate the efficiency of the LD_{90} in semi-field area against mosquito adults emergence.

MATERIALS AND METHODS

Mosquito larvae

Larvae were sampled in 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). For each sample, some larvae were separated and identified at the laboratory to confirm the species according to the methods of Hopkins (1852) and Glick (1992). In dry season, mosquitoes were sampled only at Sam-Sam III which presented more breeding sites of anopheline mosquitoes. Sampling sites included various water bodies: streams, irrigation canals, and temporary water. Larvae were collected and transported in jars containing water breeding site. At the laboratory, 3rd and 4th instars were identified and separated to the other. A temperature of 26 ± 2°C and relative humidity of 75 ± 4% was maintained. Larvae collected were used in laboratory and semi-field conditions to evaluate the inhibition of adult emergency.

Fungal formulation

Strain of *M. anisopliae* was isolated from *Oedaleus senegalensis* Krauss (Orthoptera: acrididae), at the Laboratory of Reproductive Biology. The Neem oil formulation of the fungus used for this analysis has been presented by Seye et al. (2012). The "Suneem" is emulsifiable neem oil formulated with a biodegradable solvent, Tetrahydrofurfuryl Alcohol (THFA). After determination of spore content with a Haemocytometer counter (Thoma model) dilution with 500 ml of sterile distilled water to obtain a final dose of 6×10^7 spores / ml was done. The percent (v/v) of neem oil in formulation was 0.02%

Laboratory tests

100 larvae (50 for both 3rd and 4th instars) were placed in plastic bottle (7×7×10 cm) previously sterilized at 110°C and containing 500 ml distilled water. 5 bottles were used to determine the LD_{50} and LD_{90} with the formulation at 4, 6, 8, 10 and 12% (v/v). During bioassays, the larvae were fed with bread powder mixed with fish food "Tetra WaferMix". The dead larvae and possible pupae were removed every 24 h from the bottles. After rinsing three times with distilled water to eliminate non-attached conidia, they were observed individually under magnifying microscope (× 400) to examine the fungal infection (adhesion of conidia or mycelial germination).

Semi-field treatment

The artificial breeding sites were located outside close to the laboratory of the Department of Animal Biology. Treatments were carried for two periods (rainy and dry seasons). Five trial pools with larger containers (50 x 50 x 30 cm) were selected for each treatment. 400 *A. gambiae* larvae were placed in each vat containing rain water (in rainy season) or tap water (in dry season). In dry season, leaf litters from the environmental tests are left in the vats to simulate the water conditions of breeding site which are removed just before placing larvae. Vats were covered with netting cage (50 x 50 x 20 cm) to avoid laying eggs from other mosquito species. Depth of water were conserved at 20 cm at the general breeding sites of *A. gambiae* The pools were monitored for 24 h before field treatment to allow adaptation of larvae to the new environmental conditions.

With a hand sprayer, into the four vats containing 400 larvae (3rd and 4th instars) the selected dose (LD_{90}) and four others with 0.02% (v/v) of Suneem were applied. Two vats were not treated and served as water controls. Each vat was covered with mosquito netting to trap the adults who will eventually emerge. The cumulative emerged mosquito adults were recorded for 8 days after the treatment when no larvae and pupae are still alive in the vats. The four trials used for each product represent the replicate and the results are an average. The two treatments carried out on either rainy or dry season were done depending on the availability of *A. gambiae* larvae in breeding sites.

Table 1. LD_{50} and LD_{90} of *Metarhizium anisopliae* formulated with Suneem against *Anopheles gambiae* larvae in 24 and 48 h at laboratory conditions.

Times (h)	Lethal doses (spores/ml)				
	LD_{50}	LD_{90}	R^2	Equation line	P-value
24	4.4×10^6	-	0.8337	Y=1.44 x + 0.10	< 0.0001
48	3.1×10^6	5.3×10^6	0.9757	Y= 1.69 x + 1.79	< 0.0001

Data management and statistical analysis

The larval mortality (%) observed in laboratory condition was corrected with Abbott's formula (Abbott, 1925). The relationship between probit and log concentration was established using probit equations to determine the LD_{50} and LD_{90} including Statistica 9 software and the relation formular:

$\ln (p/1-p) = \beta_0 + \beta_1 \times \ln (dose)$ (Dagnelie, 1970).
$ß_0$ and $ß_1$ are the coefficients provided by the software.

For field trials, mean emergence (E) was calculated on the basis of the number of third and forth stage larvae treated. Percent emergence (E %) was calculated using the formula :

(%E = T x 100/C) regarding WHO (WHO, 2005).
where T = emergence in treated trial and C = emergence in the control.
T-test was used to assess the efficiency between treated and none treated larvae and between two periods (rainy and dry seasons). Results were considered not statistically different at $p > 0.05$.

RESULTS

Laboratory tests

At 24 h post treatment with the formulation, LD_{50} was only obtained at 4.4×10^6 spores/ml. LD_{50} at 48 h was lower (3.1×10^6) while LD_{90} was 5.3×10^6 (Table 1). During the laboratory bioassays, few pupae emerged in lower doses but were not able to transform into adults and so die within 24 h.

Microscopic observations

Spores were found on the dead larvae (cuticle, abdomen and the antenna) 24 h after fungal treatment (arrows on Figures 1A and B). The hyphal development was more effective at 48 h (arrows on Figure 1C) and 72 h (Figure 1D) with apparition of germ tube and early hyphal sporulation. Conidial attack was observed on the pupae around the thorax and paddles (Figures 2A and B). This led to the dead of pupae by stopping the processes of adult emergency.

Field treatment

M. anisopliae oil formulation (OF) in semi-field area (Figures 3 and 4) inhibited the emergence of adults mosquitoes. The mosquito collected by emergence traps increased at the control vat for the two treatment periods. This inhibition was more effective with the *M. anisopliae* oil formulation (OF) than with suneem alone (S) or control. After spraying with LD_{90} (5.3×10^6 spores/ml), the emergence rate at day 8 in the rainy period were 1.25 ± 0.15, 30.25 ± 1.23 and 98 ± 0.76 % for the oil formulation (OF), Suneem (S), and control, respectively. The mean environmental temperatures for water was between 25 and 28°C.

In dry season, the cumulative emergence percent at day 8 were 2.25 ± 0.03, 28 ± 1.07 and 97.25 ± 1.56% for the oil formulation (OF), Suneem (S), and control respectively. Environmental and water temperatures were 27 and 24°C respectively. The percent of adult emergence were not statistically different between rainy and dry seasons (p= 0.3). Nevertheless, suneem oil was also effective against adult emergence (Figures 3 and 4).

After treatment, it was found that some dead adults resting on the water surface had fungus germinating around them as well as on some dead larvae and pupae.

DISCUSSION

Currently, entomopathogenic fungus such as *M. anisopliae* against mosquitoes is one of the most promising method in mosquito vector control. The fundamental idea of using *M. anisopliae* in oil formulation against mosquitoes species, is the pathogenicity regarding infection of larvae or adults. We have recently shown that *M. anisopliae* formulated with suneem (emulsifian oil neem) was effective against *A. gambiae* adults (Seye et al., 2012). In this present study, it was showed that, this formulation is also effective against *A. gambiae* mosquito larvae in laboratory and semi-field conditions. This effectiveness, favored by suneem formulation has been manifested by the inhibition of mosquito emergence at dry and rainy seasons. Also, it was supposed that Suneem used for formulation, protects conidia from adverse environmental conditions and facilitates spray and adhesion to the insect cuticle. Also neem products have already revealed their effectiveness in larvicidal effect (Vatandoost et al. 2004) and pupal death (Seye et al., 2006) even if the percent of Suneem used in

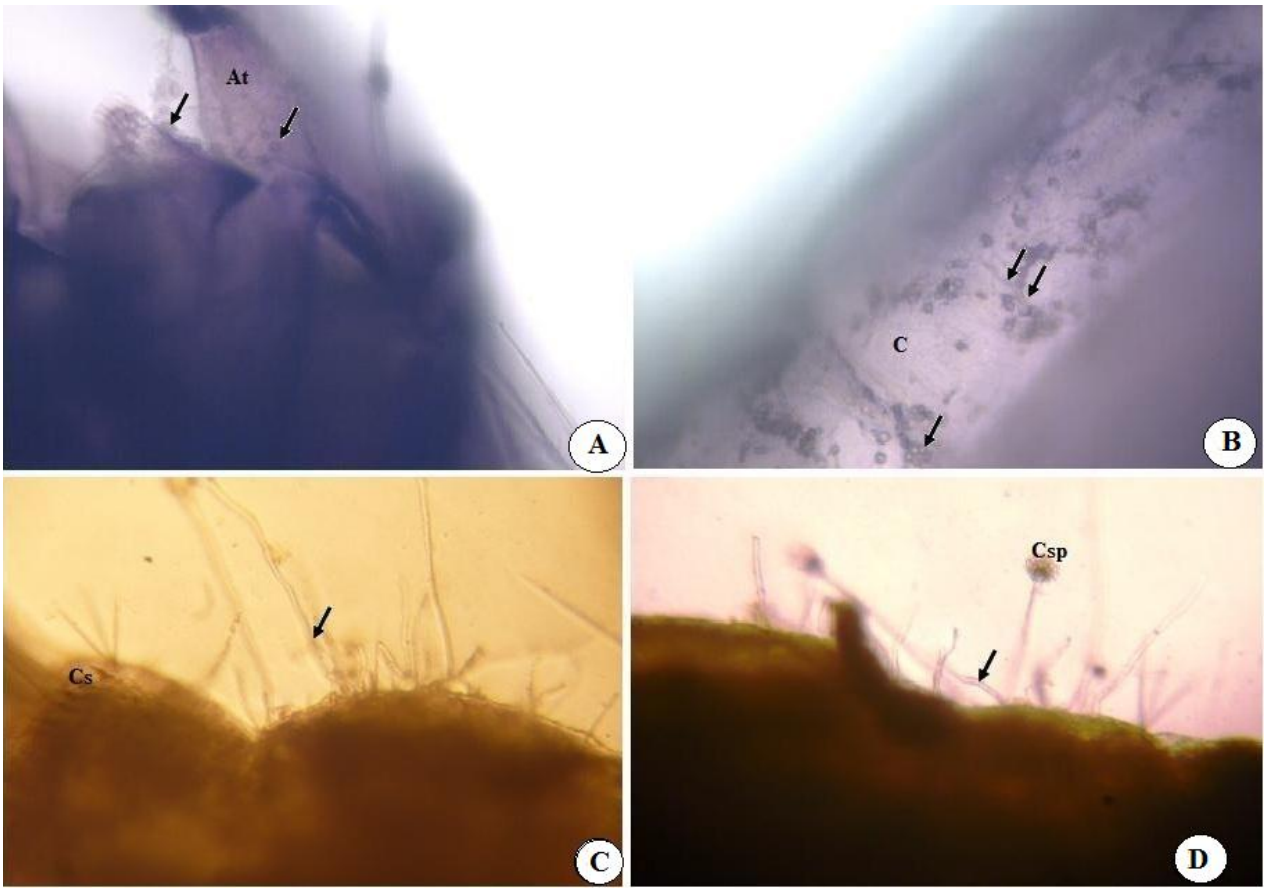

Figure 1. *Anopheles gambiae* larvae infected by *Metarhizium anisopliae* formulated with neem oil (Suneem) 24 h (A and B), 48 h (C**)** and 72 h (D) after treatment (x 400). C= cuticle, At. = antenna, Csp= conidial spore, Cs= comb. Conidia were adhered (arrows) on the head (A) and abdomen (B) in 24 h. Mycelia germination was observed on the larval cuticle in 48 h and 72 h (arrows).

this present study was lower (0.02%) than emulsible oil above 0.5% used by Depieri et al. (2005). Some authors have found low efficacy from the combination of neem oils with fungi (Bajan et al., 1998; Hirose et al., 2001; Depieri et al., 2005), although not all (Visalakshy et al., 2006; Rodrigues-Lagunes et al., 1997; Seye et al., 2008). But with neem seed extract in concentrations above 2.5%, the fungitoxic effects could be observed (Rodrigues-Lagunes et al., 1997). Therefore, it is possible to formulate entomopathogenic fungi as *M. anisopliae* regarding the neem oil content for insect control. In this context, Sahayaraj et al. (2011) showed useful information on the compatibility between the fungal biological control agents with plant-based insecticides and plant extracts which are commonly used in pest management. Therefore, a combination of entomopathogenic fungi with plant based insecticides may provide also a more sustainable management strategy.

The fungal germination around immature mosquito is very effective against adult emergence. Our results revealed that, the percent of adult emergent was higher in untreated larvae than treated mosquitoes with *M. anisopliae*. Indeed, the magnifying microscope reveals that, at 24 and 48 h after treatment, larvae were infected by *M. anisopliae* conidia via cuticle attack and mycelium development around the mosquito larvae. This infection was also observed on dead pupae. The 4th stage larvae were not transformed to pupae and were killed during their transformation into pupae or during adult emergence. In our previous study, it was showed that mosquito was infected by the fungi *Aspergillus clavatus* (Desmazières) through direct contacting with the cuticle or by ingestion (Seye et al., 2008). In general, the fungal conidia penetrate the insect cuticle and grow into the haemocoele where they produce a blend of organic compounds, causing internal mechanical damages, nutrient depletion (Gillespie and Clayton, 1989), resulting in mycosis and death (Clarkson and Chrarnley, 1996). But also, microscope observation revealed that pupal development was arrested resulting in decreased pupal transformation and death. This is consistent with other results (Bukhari et al., 2011).

Even if reduced exposure time can influence the control potential of fungus, the amount of nutrients in the breeding sites and larval density are known to have impact on larval

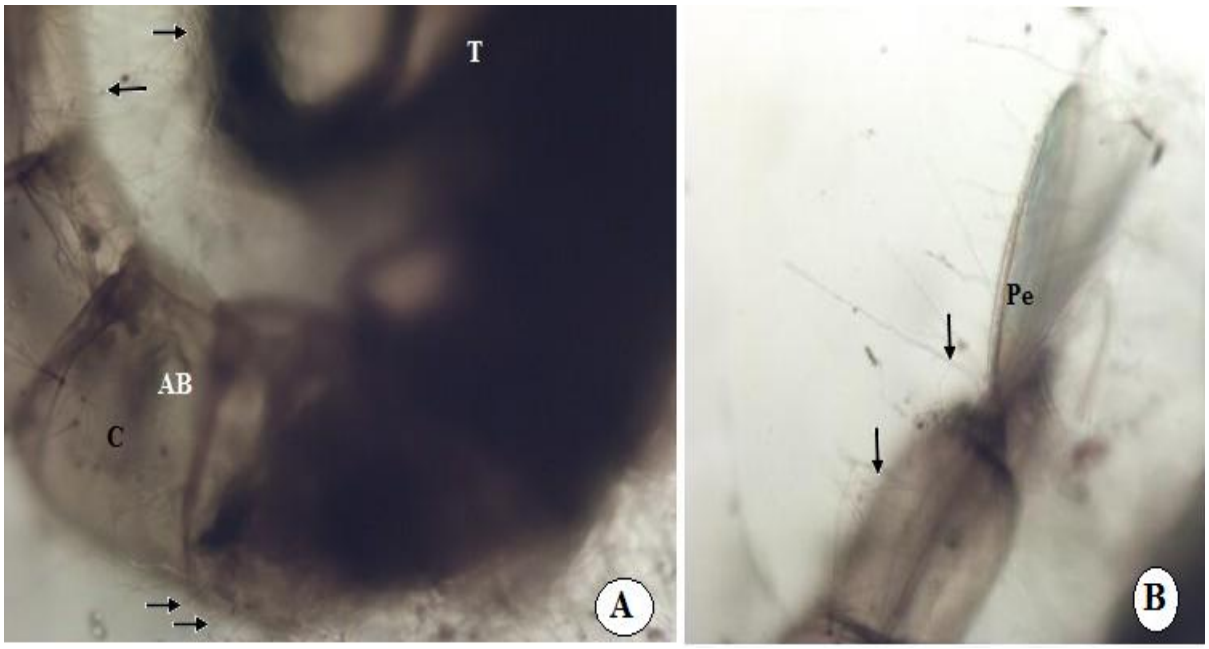

Figure 2. *Anopheles gambiae* pupae infected by *Metarhizium anisopliae* formulated with neem oil (Suneem) 48h after treatment (A and B) (x400). AB= abdomen, C= cuticle, Pe= paddles, T= thorax. Mycelia germination was observed on the pupae (arrows) at the thorax and abdomen.

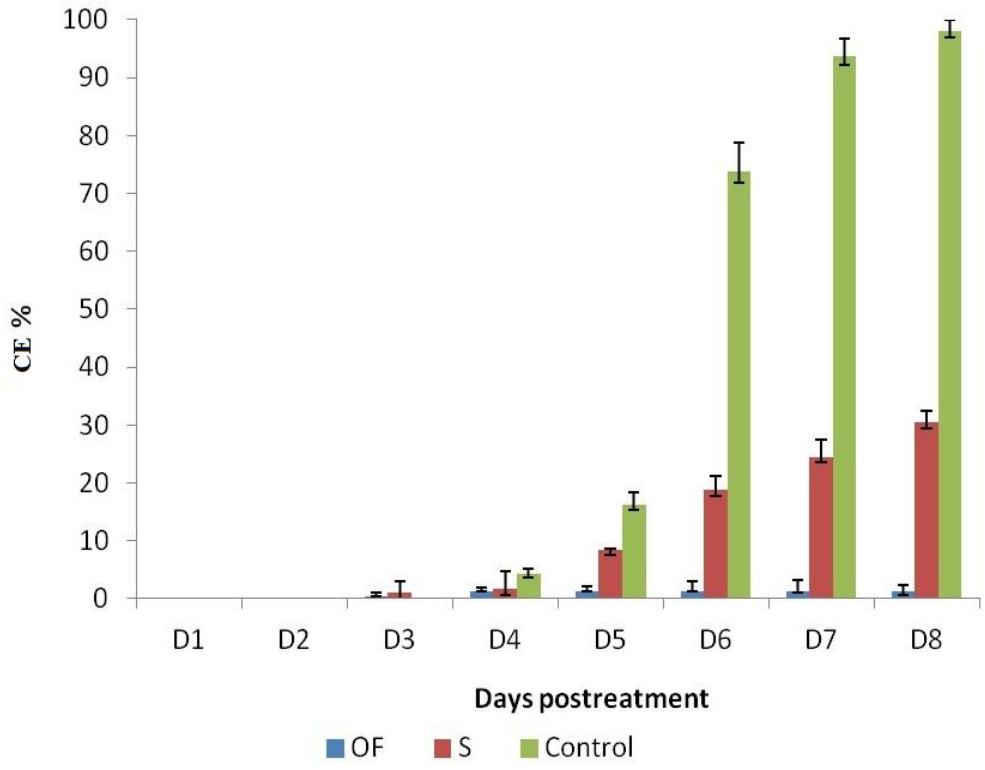

Figure 3. Percent of cumulative emergence of *Anopheles gambiae* s.l. adults treated with *Metarhizium anisopliae* oil formulation in rainy season. The environmental temperature mean was 28 °C and 25 °C for the water vats. Percent of emergence inhibition are more significant with oil formulation than suneem (P= 0.02) or control (P<0.0001). CE= cumulative emergence, OF= oil formulation, S= Suneem.

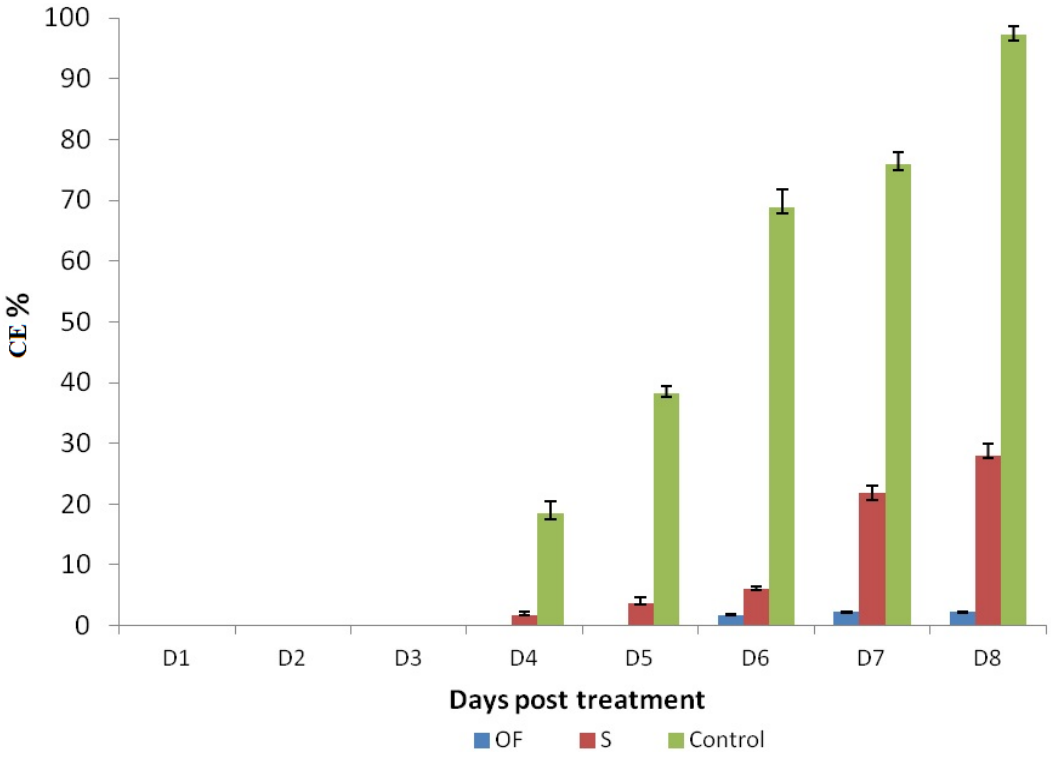

Figure 4. Percent of cumulative emergence of *Anopheles gambiae* s.l. adults treated with *Metarhizium anisopliae* oil formulation in dry season. The environmental temperature mean was 27°C and 24°C for the water vats. T-test reveal that Percent of emergence inhibition are more significant with oil formulation than Suneem (P=0.04) or control (P<0.0001), CE= cumulative emergence, OF= oil formulation, S=Suneem.

survival (Koenraadt et al., 2004; Bukhari et al., 2011). Pelizza et al. (2007) showed that larvae at higher density showed low mortality due to reduced spore-share per larvae. In our study, the larval density was 0.16 larvae /cm^2. The formulation with emulsible oil allowed the conidia to be in contact with larvae and to be ingested, it is not in this case that they are applied with the dry formulation, because they clump together in an aquatic environment, reducing the contact area with mosquito larvae at the water surface.

Without protection, fungal spores are sensitive to temperature, humidity and ultraviolet radiation. High relative humidity triggers germination of spores and is therefore likely play a negative role when spores are applied over the water surface (Zimmermann, 2007). But under field conditions, the environmental factors recorded in our study (temperature and relative humidity) were not apparently critical to the fungal pathogenicity. However, the influence of environmental factors needs to be evaluated for conidial tolerance in the formulation. Conidial resistance could be of benefit in field environment for the persistence of the conidia, favoring virulent spores but also along with mosquito adult exposition when female are in oviposition.

In our study, *M. anisopliae* formulated with suneem was effective against *A. gambiae* larvae for both periods (dry and rainy seasons). The *M. anisopliae* strain used has practically the same effectiveness against *A. gambiae* emergence for both periods.

Thereby, this formulation could be used against mosquito adult and aquatic stages. This will be of benefit for more sustainable management strategy and reduced cost for mosquito vector control and malaria elimination.

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