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# Activity of additives and their effect in formulations of *Metarhizium anisopliae* s.l. IP 46 against *Aedes aegypti* adults and on *post mortem* conidiogenesis

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ABSTRACT

*Background:* Oil formulations of entomopathogenic fungi have interest for biological mosquito control.
*Objectives:* The activities of *M. anisopliae* s.l. IP 46 conidia were tested in *Aedes aegypti* adults either without any formulation or formulated with vegetable or mineral oil and in combination with diatomaceous earth.
*Findings:* IP 46 was highly active against adults, the vector of important arboviruses in the tropics and subtropics. At an exposure of adults to $3.3 \times 10^7$ conidia/cm$^2$, values of lethal times $TL_{50}$ and $TL_{90}$ reached minimal 3.8 and 4.6 days, respectively, and lethal concentrations $LC_{50}$ and $LC_{90}$ were $2.7 \times 10^5$ and $2.4 \times 10^6$ conidia/cm$^2$, respectively, after 10 days of exposure. Activity against adults was improved by diatomaceous earth (KeepDry® KD) combined with mineral oil (Naturol® N) or vegetable oil (Graxol® G). Additives KD or N separately (and G to a lesser extent) or in combination, KD + N and KD + G without conidia had also a clear adulticidal effect. Efficacy of conidia formulated or not with KD + N decreased somewhat at shorter exposure periods. Time of exposure (0.017, 12, 48, 72 or 120 h) of adults to KD and N or IP 46 or conidia and KD and N had no significant effect on mortality. *M. anisopliae* s.l. recycled on fungus-killed mosquitoes producing high quantities of new conidia regardless of the conidial concentrations or formulations tested. Additives tested had no clear effect on quantitative conidiogenesis on cadavers.
*Main conclusions:* Formulations of IP 46 conidia with mineral oil and diatomaceous earth represent a promising tool for the development of potent strategies of focal control of this important vector with entomopathogenic fungi.

## 1. Introduction

*Aedes aegypti* (Diptera: Culicidae) is the principal vector of Dengue, Chikungunya, Zika and Mayara fever in the tropics and subtropics, and a key target for vector control of arbovirus infection (Weaver et al., 2018). This mosquito is highly adapted to human urban life and common in domestic and peridomestic areas. Females feed on their hosts during the day and proliferate in small or medium-sized breeding sites. When not active both females and males rest in the same areas. In the tropics, populations of *A. aegypti* persist throughout the year, even during the dry season, and number of vectors rises dramatically in the rainy season increasing the risk of epidemic outbreaks of mentioned viral diseases. The effective control of this vector is difficult due to the high resistance against synthetic insecticides, skip oviposition behavior, flexibility of breeding site selection and a high resistance of eggs to desiccation (Reiter, 2007; Wong et al., 2011; Moyes et al., 2017; Garcia et al., 2018).

Hypocrealean fungi from the genus *Metarhizium* have high potential for biological control of mosquito vectors of human diseases (Scholte et al., 2004; Lacey, 2017; Mascarin et al., in press; Thomas, 2018). These fungal entomopathogens generally infect their hosts through the cuticle and then develop in the hemolymph and inner organs. Dynamics of invasion are determined by the specific virulence of the fungal pathogen, a high initial inoculum of infective propagules (generally conidia) on the cuticle, and favorable high ambient humidity for extracuticular development of the entomopathogen. After infection hosts eventually succumb in the following days, and depending on ambient moisture, the fungus emerges on the dead host and produces new infective conidia.

IP 46, a member of the *Metarhizium anisopliae* complex, has been

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studied over many years against invertebrates with medical and veterinary importance (Rocha and Luz, 2011; Luz et al., 2012, 2016; Hubner-Campos et al., 2013; Duarte et al., 2015; Gutierrez et al., 2016). This strain also has promising activity against eggs and larvae of *A. aegypti* (Silva et al., 2004; Albernaz et al., 2009; Santos et al., 2009), and there is initial evidence of adulticidal activity in this vector (Leles et al., 2010; Falvo et al., 2016). IP 46 was isolated from a soil sample in Central Brazil in 2001 (Rocha et al., 2013).

Additives in fungal formulations intend to improve the effect of the pathogen in the target pest. Mineral and vegetable oils applied on pest insects may affect insects and are often used in fungal formulations. Conidia of *Metarhizium* spp. formulated in pure oil or in oil-in-water formulations and applied on the cuticle adhered to the cuticle in higher numbers and more homogenously than conidia without oil. In addition, oil-formulated propagules developed quicker on the cuticle than unformulated conidia. Oils may also protect propagules after application or after adhesion on the cuticle against harmful abiotic stresses (Burges, 1998; Luz and Batagin, 2005; Barreto et al., 2016; Alves et al., 2017). The activity of oil-formulated *Metarhizium* conidia against arthropods was distinctly improved at suboptimal conditions of ambient humidity, challenging high temperatures or UV radiation for germination of conidia (Moore et al., 1993; Inglis et al., 1995; Morley-Davies et al., 1996). Diatomaceous earth, which is known to cause abrasive damage to the cuticular surfaces, is a known insecticide against arthropod pests of stored grains (Korunic, 1998; Akbar et al., 2004; Dal Bello et al., 2006; Athanassiou and Steenberg, 2007; Batta, 2008) and as an additive, especially in combination with oil-enhanced fungal activity of fungal entomopathogens, against triatomine vectors (Luz et al., 2012).

Oil formulations of *M. anisopliae* have been tested against mosquito adults, especially *Anopheles* spp. (Scholte et al., 2006; Mnyone et al., 2009, 2011), but little is known yet about the potential of these and other additives against *A. aegypti* (Paula et al., 2008; Carolino et al., 2014). We report here on the toxicity of vegetable and mineral oil and diatomaceous earth and their effects as additives to fungal formulations of IP 46 against *A. aegypti* adults and on conidiogenesis on fungus-killed individuals.

## 2. Materials and methods

### 2.1. Origin, rearing and preparation of mosquitoes

The colony of *A. aegypti* originated from larvae collected in Goiânia, Brazil, in 2012, and was maintained in the laboratory at 27 ± 5 °C, 75 ± 10% relative humidity (RH) and natural photophase. Adults were fed *ad libitum* on cloth pads imbibed with 10% sucrose solution. Females were fed twice a week following the method described by Lima et al. (2009), a technique approved previously by the Ethics Commission for the Use of Animals of the Federal University of Goiás, Goiânia (CEUA 079/13, UFG, February 10, 2014). Eggs were prepared according to Rocha et al. (2015). For assays, filter papers with eggs were transferred to a plastic bowl (29 cm diameter, 15 cm height) with 1.000 ml of tap water. Larvae were fed with triturated cat food (Bom Preço®, Salto de Pirapora, Brazil) until pupation, and pupae then transferred to a plastic cup (50 ml) containing 30 ml of tap water, and the cup placed in a plastic container (500 ml) covered with a mesh to collect emerging adults.

### 2.2. Origin and preparation of the fungus

*M. anisopliae* s.l. IP 46 from Central Brazil is stored in the Fungal Collection of IPTSP and as CG 620 at Embrapa Genetic Resources and Biotechnology, Brasília, Brazil. Before the tests, the isolate was host-passaged on adult *A. aegypti* as reported by Luz et al. (2007) to standardize its virulence. The isolate was cultivated on PDA (potato-dextrose-agar, Acumedia, Lansing, Michigan, USA) at 25 ± 1 °C, 75 ± 10% RH and 12 h photophase for 15 days. Aerial conidia were harvested directly by scraping with a spatula from the surface of the culture, transferred to a Petri dish and dried on silica gel for 5 days at 4 °C. For the quantification of the conidia, 5 mg of dried conidia were suspended in 1 ml of sterile 0.01% Tween 80 (polyoxyethylene sorbitan monoleate), and the number of conidia (conidia/mg) calculated based on hemacytometer counts. The viability of conidia (> 95% germination) was assessed at the beginning of each test inoculating 100 µl of a conidial suspension at $10^5$ conidia/ml onto SDAY (Sabouraud-Dextrose-Yeast-Agar) medium. Germination of 400 conidia was quantified with a light microscope, after 20–24 h incubation at 25 ± 1 °C and 12 h photophase. Conidia were considered germinated if their germ tubes were longer than the conidial diameter.

### 2.3. Additives and their application

The inner surface of transparent plastic cups with lids (Copocentro, Anapolis, Brazil, 10.5 cm height, 7 cm diameter with a total inner surface area of 336 $cm^2$) was roughened with sandpaper (A-257, G220, Norton Saint-Gobain Abrasivas, Guarulhos, Brazil) to permit a homogenous distribution of additives and conidia on the surface. About 45 regularly distributed circular openings (≤ 1 mm diameter) in the cup provided a permanent air exchange. Emulsifiable vegetable oil (Graxol®, Agrária Indústria e Comércio Ltda., Jardinópolis, Brazil) mentioned in the following as G was tested at 0.25 µl/$cm^2$. Mineral oil (Naturol®, Farmax Ltda., Divinópolis, Brazil) indicated as N was tested at 0.25 µl/$cm^2$, and diatomaceous earth (KeepDry®, Irrigação Dias Cruz Ltda., Santo André, Brazil) mentioned as KD was tested at 0.6 mg/$cm^2$. The oils were distributed manually using the index finger protected by a sterile latex surgical glove (Talge® Descartáveis do Brasil Ltda., Itajaí, Brazil), and KD and conidia with a paintbrush on the whole inner surface of the cup. Additives (G, N or KD) were tested separately, in combination (G and KD or N and KD) with or without conidia ($3.3 \times 10^6$ conidia/$cm^2$); finally, conidia without any additives ($10^5$, $10^6$, $3.3 \times 10^6$, $10^7$ and $3.3 \times 10^7$ conidia/$cm^2$), and a control group with neither conidia nor additives were also included.

### 2.4. Evaluation of adulticidal activity

Ten *A. aegypti* adults, aged to 24–72 h after emergence, were transferred to each treated cup and exposed to additives and/or conidia for 60 sec (0.017 h), 12, 48, 72, 120 h or permanently, and then incubated at 25 ± 1 °C, 75 ± 5% RH and 12 h photophase for up to 15 days. Adults were able to feed *ad libitum* on a strip of filter paper adjusted in a small glass vial (2 cm diameter and 4.5 cm height) filled with previously sterilized 10% aqueous sucrose solution and replaced on alternating days. Mortality was monitored daily, and dead mosquitoes retrieved and processed for further conidiogenesis as explained in the following. Routinely, four independent repetitions were carried out.

### 2.5. Quantification of conidia on mycotized adults

Dead adults were incubated on filter paper at 25 ± 1 °C, > 98% RH and 12 h photophase for 10 days, and fungal development was checked daily. Adults were then transferred individually to a tube with 1 ml 0.01% Tween 80 and vortexed for 3 min. The conidial suspensions were diluted as necessary, and the total number of conidia determined based on hemacytometer counts.

### 2.6. Analysis of data

Data percentages were arcsine-square root transformed and then analyzed with analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK) multiple range test for comparison of means. Means were considered statistically different at $P < 0.05$. Lethal concentrations ($LC_{50}$ and $LC_{90}$) and times to kill 50% and 90% ($LT_{50}$ and $LT_{90}$) of mosquitoes were calculated by probit analysis of independent and

**Table 1**
Cumulative relative mortality ( ± standard error, SE) after a 5 and 15 day exposure and lethal time (days) to kill 50 and 90% ($LT_{50}$ and $LT_{90}$ with their respective 95% confidence interval, CI, slope ± SE) of adult *Aedes aegypti*, exposed to *Metarhizium anisopliae* s.l. IP 46, and the mean number of conidia ( ± SE) found on adults*.

| Conidia/cm$^2$ | Cumulative mortality | | Lethal time | | | Number of conidia/adult ± SE*** |
|---|---|---|---|---|---|---|
| | 5 days | 15 days | $LT_{50}$ (CI) | $LT_{90}$ (CI) | Slope ± SE | |
| 0 | 0 a | 0 a | ** | ** | – | – |
| $10^5$ | 7.5 ± 3 a | 75 ± 3.2 b | 11.7 (9.8–14) c | 19.3 (16.6–23.6) b | 0.2 ± 0.02 | 4.9 ± 1.4 a |
| $10^6$ | 45 ± 4.4 b | 100 c | 7.5 (5.3–9.5) b | 12.4 (10.3–16.5) b | 0.3 ± 0.03 | 8.7 ± 3.7 a |
| $3.3 \times 10^6$ | 20 ± 4.6 a | 100 c | 7.2 (4.2–9.9) bc | 11.9 (9.4–18.7) b | 0.3 ± 0.04 | 7.6 ± 1.6 a |
| $10^7$ | 77 ± 6 c | 100 c | 4.2 (0.5–6.9) ab | 7.2 (5.2–17.5) b | 1.7 ± 0.25 | 8.7 ± 1 a |
| $3.3 \times 10^7$ | 97 ± 1.7 d | 100 c | 3.8 (3.6–4) a | 4.6 (4.3–4.9) a | 0.3 ± 0.07 | 13.4 ± 2.3 a |
| $F_{5,18}$ | 47.4 | 32 | – | – | | $F_{4,10}$ 1.3 |
| *P* | < 0.001 | < 0.001 | – | – | | *P* = 0.4 |

Ten adults were exposed in a plastic cup with a 336 cm$^2$ total inner surface area, previously untreated (control) or treated with conidia at different concentrations and incubated at 75 ± 5% relative humidity (RH) and 25 ± 1 °C up to 15 days. Values (calculated with four independent repetitions) within the same column (mortality and numbers of conidia with ANOVA and SNK-test at $P < 0.05$; $LT_{50}$ and $LT_{90}$ with Probit analysis on their CI 95%), followed by different letters (a–d) were significantly different among each other.
*dead adults were incubated at 25 ± 1 °C and > 98% RH for 10 days; **cumulative mortality was too low (≤ 50%) to calculate $LT_{50}$ and $LT_{90}$; ***all values multiplied by $10^6$.

dependent values, respectively (Preisler and Robertson, 1989; Throne et al., 1995).

## 3. Results

### 3.1. Adulticidal activity of IP 46 and development on dead adults

All adults exposed to IP 46 conidia, without any further additive, at 25 ± 1 °C and 75 ± 5% RH survived the first 2 days, regardless of the conidial concentrations tested ($10^5$ up to $3.3 \times 10^7$ conidia/cm$^2$). The first dead adults were detected after a 3-day incubation at ≥ $10^7$ conidia/cm$^2$, and first dead mosquitoes were found after the 4$^{th}$ day for the other concentrations ($10^5$ up to $3.3 \times 10^6$ conidia/cm$^2$). Mortality increased in the next days, and there was a highly significant effect of the conidial concentration on cumulated mortality at a 5- and 15-day exposure ($F_{5,18} \geq 32$; $P < 0.001$; Table 1). Values of $TL_{50}$ and $TL_{90}$ decreased at higher conidial concentrations and reached minimal 3.8 days ($TL_{50}$) and 4.6 days ($TL_{90}$) (Table 1). Survival time was highest at lowest conidial concentrations tested ($10^5$ conidia/cm$^2$) with $TL_{50}$ 11.7 days and $TL_{90}$ 19.3 days (Table 1). Lethal concentrations to kill 50% ($LC_{50}$) and 90% ($LC_{90}$) of the adults were $5.8 \times 10^6$ and $1.7 \times 10^7$ conidia/cm$^2$ after 5 days of exposure and $2.7 \times 10^5$ and $2.4 \times 10^6$ conidia/cm$^2$ after 10 days of exposure (Table 2). No mortality was found in the control groups in any repetition up to 15 days of exposure.

Mycelium and conidia were detected on dead adults, 2 and 4 days after exposure in a moist chamber, respectively, regardless of the conidial concentration to which mosquitoes were previously exposed. The mean number of newly produced conidia found on dead adults after a 10-day incubation varied between $4.9 \times 10^6$ conidia/adult (at initially $10^5$ conidia/cm$^2$) and $13.4 \times 10^6$ conidia/adult (at initially $3.3 \times 10^7$ conidia/cm$^2$) without significant effect of the conidial concentrations tested on quantitative conidiogenesis on dead mosquitoes ($F_{4,10} = 1.3$; $P = 0.4$; Table 1).

**Table 2**
Lethal concentration (conidia/cm$^2$) to kill 50 and 90% ($LC_{50}$ and $LC_{90}$ with their respective 95% confidence interval, CI, slope ± standard error, SE) of adult *Aedes aegypti*, exposed to *Metarhizium anisopliae* s.l. IP 46.

| Days | $LC_{50}$ (CI) | $LC_{90}$ (CI) | Slope ± SE |
|---|---|---|---|
| 5 | $5.8 \times 10^6$ ($2 \times 10^6$–$1.3 \times 10^7$) a | $1.7 \times 10^7$ ($9.2 \times 10^6$–$7.1 \times 10^7$) a | 2.7 ± 0.11 |
| 10 | $2.7 \times 10^5$ ($1.3 \times 10^5$–$4 \times 10^5$) b | $2.4 \times 10^6$ ($1.5 \times 10^6$–$4 \times 10^6$) b | 1.1 ± 0.13 |

Ten adults were exposed in a plastic cup with a 336 cm$^2$ total inner surface area, previously treated with conidia at different concentrations ($10^5$–$3.3 \times 10^7$ conidia/cm$^2$) and incubated at 75 ± 5% relative humidity and 25 ± 1 °C up to 10 days. Values (calculated with four independent repetitions) within the same column (based on their CI 95%), followed by different letters (a–b) were significantly different among each other. No control mortality up to a 10-day exposure in untreated cups was recorded.

### 3.2. Activity of additives and formulations

First adults died within 3 days of exposure (IP 46 + KD and IP 46 + KD + N) or 4 days (IP 46 and IP 46 + KD + G), and at a 5-day exposure there was a significant effect of the formulation on cumulative mortality ($F_{11,36} = 2.3$; $P = 0.03$), that varied between 0% (G) and 30% (N and IP 46 + KD + G; Table 3). Mortality increased in the next days with the exception of the control, and at a 15-day exposure the effect of the formulation on cumulative mortality was highly significant ($F_{11,36} = 16.3$; $P < 0.001$) with highest mortality (100%) testing unformulated conidia or conidia formulated only with KD or KD + N or KD + G. Whereas no control mortality and a low cumulative mortality of adults treated with G (17.5%) were observed, mortality of adults tested with KD or N was ≥ 77.5% at the same moment (Table 3). The smallest values of $LT_{50}$ (≤ 6.4 days) were found for conidia formulated with KD or KD + G or KD + N, and the smallest values of $LT_{90}$ (≤ 7.9 days) testing conidia formulated with KD + N or KD + G (Table 3).

The number of newly produced conidia on dead adults after a 10-day incubation varied between lowest $2.8 \times 10^6$ conidia/adult (N) and highest $6.3 \times 10^6$ conidia/adult (KD) with a highly significant effect of the formulation tested on overall new post-mortem conidiogenesis ($F_{5,139} = 12$; $P < 0.01$; Table 3).

### 3.3. Evaluation of different exposure times of Aedes aegypti

Time of exposure (0.017, 12, 48, 72 or 120 h) of adults had no significant effect on mortality ($F_{14,45} \leq 1.7$; $P \geq 0.2$) up to 5 days in cups treated only with KD (0.6 mg/cm$^2$) and N (0.25 µl/cm$^2$) or IP 46 ($3.3 \times 10^6$ conidia/cm$^2$) or conidia and KD and N at the same concentration. Cumulative relative mortality after a 5-day exposure varied between 0% (adults exposed for 120 h to cups treated with KD + N) and 38% (adults exposed for 120 h to cups treated with IP 46 + KD + N; Table 4). Mortality increased in the following days and, after a 15-day exposure, reached total mortality in cups treated with IP 46 only and a previous 120 h exposure of adults to conidia and with IP 46 formulated with KD + N at a 72 or 120 h exposure. Cumulative mortalities within 15 days were lowest (42.5–82.5%) testing additives

**Table 3**
Cumulative mortality ( ± standard error, SE) after a 5 and 15 day exposure, lethal time (days) to kill 50 and 90% ($LT_{50}$ and $LT_{90}$ with their respective 95% confidence interval, CI, slope ± SE) of adult *Aedes aegypti*, exposed to single additive or combined formulation of *Metarhizium anisopliae* s.l. IP 46, and the mean number of conidia ( ± SE) found on adults*.

| Additive or formulation | Cumulative mortality | | Lethal time | | | Number of conidia/adult ± SE*** |
|---|---|---|---|---|---|---|
| | 5 days | 15 days | $LT_{50}$ (CI) | $LT_{90}$ (CI) | Slope ± SE | |
| Control | 0b | 0c | ** | ** | – | – |
| KD | 7.5 ± 4 ab | 87.5 ± 8.2 a | 10.4 (8.6–12.2) b | 17.2 (14.9–20.6) c | 0.2 ± 0.02 | – |
| N | 30 ± 15 a | 77.5 ± 14 a | 9.7 (0–30.7) ab | 19.8 (12.4–179.8) c | 0.1 ± 0.02 | – |
| G | 0 b | 17.5 ± 6 b | 17.6 (15.9–30.8) b | 21.9 (18.1–49.7) c | 0.3 ± 0.1 | – |
| KD + N | 7.5 ± 4 ab | 92.5 ± 4 a | 8.9 (7.7–10.2) ab | 13.9 (12.5–18.9) c | 0.3 ± 0.03 | – |
| KD + G | 7.5 ± 2 ab | 97.5 ± 2 a | 9.8 (7.9–11.9) ab | 15.1 (12.9–18.9) c | 0.2 ± 0.03 | – |
| IP 46 | 22.5 ± 6 a | 100 a | 6.4 (5.7–7) a | 9 (8.2–10.1) b | 0.5 ± 0.06 | 5 ± 0.3 b |
| IP 46 + KD | 17.5 ± 9 a | 100 a | 5.8 (4–8) a | 7.9 (6.4–14.6) ab | 0.6 ± 0.08 | 6.3 ± 0.1 c |
| IP 46 + N | 7.5 ± 4 ab | 85 ± 6 a | 10.2 (1.8–19) ab | 17.9 (12.6–54.6) c | 0.2 ± 0.02 | 2.8 ± 0.1 a |
| IP 46 + G | 7.5 ± 6 ab | 75 ± 13 a | 10.2 (0.3–23.4) ab | 18.5 (12.6–87.5) c | 0.2 ± 0.02 | 3 ± 0.1 ab |
| IP 46 + KD + N | 25 ± 2 a | 100 a | 5.8 (5.4–6.3) a | 7.5 (6.9–8.3) a | 0.8 ± 0.1 | 4 ± 0.9 b |
| IP 46 + KD + G | 30 ± 9 a | 100 a | 5.9 (5.4–6.3) a | 7.6 (7–8.5) a | 0.7 ± 0.1 | 6 ± 0.2 c |
| $F_{11, 36}$ | 2.3 | 16.3 | – | – | | $F_{5,139}$ 12 |
| *P* | 0.03 | < 0.001 | – | – | | *P* < 0.01 |

Ten adults were exposed in a plastic cup with a 336 $cm^2$ total inner surface area, previously untreated (control) or treated with conidia ($3.3 \times 10^6$ conidia/$cm^2$) or with KeepDry® (KD) at 0.6 mg/$cm^2$, mineral oil Naturol® (N) at 0.25 µl/$cm^2$, vegetable oil Graxol® (G) at 0.25 µl/$cm^2$, combination KD + N or KD + G or combined with conidia of IP 46 (IP 46 + KD; IP 46 + N; IP 46 + KD + N; IP 46 + KD + G), incubated at 75 ± 5% relative humidity and 25 ± 1 °C up to 15 days. Values (calculated with four independent repetitions) within the same column (mortality based on ANOVA and SNK test at $P < 0.05$; $LT_{50}$ and $LT_{90}$ on their CI 95%), followed by different letters (a–c) were significantly different among each other.
*dead adults were incubated at 25 ± 1 °Cand > 98% RH for 10 days; **cumulative mortality was too low (≤ 50%) to calculate $LT_{50}$ and $LT_{90}$. ***all values multiplied by $10^6$.

KD + N only and increased testing IP 46 (77.5–100%) and IP 46 applied with KD + N (85–100%). Control mortality was ≤ 5% at the same time. There was a significant effect of exposure time testing unformulated conidia ($F_{4,15} = 4.4$; P = 0.01: 0.017 h < 12 h up to 120 h) but not of conidia formulated with KD + N or KD + N without conidia ($F_{4,15} \leq 2$; $P \geq 0.13$) on mortality (Table 4).

Lethal times $LT_{50}$ and $LT_{90}$ decreased with longer initial exposure times, and were shortest ($LT_{50}$ 5.2 days and $LT_{90}$ 7.8 days) after exposing adults for maximal 5 days to conidia formulated with KD + N. The longest values of $LT_{50}$ (≥ 10.5 days) and $LT_{90}$ (≥ 18.7 days) were obtained with KD + N (Table 4).

The mean numbers of new conidia produced on dead adults varied between lowest of $4.2 \times 10^6$ conidia/adult (adults previously exposed to conidia for 5 days) and the highest of $8.4 \times 10^6$ conidia/adult (previously 2 days) with a significant effect of the formulation/exposure time on quantitative conidiogenesis on dead adults ($F_{9,158} = 3.7$; P = 0.003; Table 4).

## 4. Discussion

Our findings strengthened and increased the potential for using *M. anisopliae* s.l. IP 46 against *A. aegypti* adults. After exposing adults to unformulated conidia at a high concentration ($3.3 \times 10^7$ conidia/$cm^2$), this fungus was able to kill almost all mosquitoes in fewer than 5 days, or at lower concentrations of conidia (≥ $10^6$ conidia/$cm^2$) all mosquitoes died within two weeks. A fast elimination of adult vectors by

**Table 4**
Cumulative relative mortality ( ± standard error, SE) after 5 and 15 days of exposure of *Aedes aegypti* adults to conidia of *Metarhizium anisopliae* s.l. IP 46 formulated with diatomaceous earth (KeepDry®) and mineral oil (Naturol®) and lethal time (days) to kill 50 and 90% ($LT_{50}$ and $LT_{90}$) with their respective 95% confidence interval (CI, slope ± SE), and the mean number of conidia ( ± SE) found on adults.

| Conidia | KD + N | Time of exposure (h) | Cumulative mortality | | Lethal time | | | Number of conidia/adult** ± SE |
|---|---|---|---|---|---|---|---|---|
| | | | 5 days | 15 days | $LT_{50}$ (CI) | $LT_{90}$ (CI) | Slope ( ± SE) | |
| – | + | 0.017 | 10 ± 7.5 | 42.5 ± 22.5 a | * | * | – | – |
| – | + | 12 | 30 ± 15.4 | 67.5 ± 12 ab | 11.4 (3–26.7) bc | 25 (16.1–82) b | 0.1 ± 0.02 | – |
| – | + | 48 | 17.5 ± 5.4 | 82.5 ± 7.4 ab | 10.5 (6.1–16) abc | 18.7 (14–33) b | 0.1 ± 0.03 | – |
| – | + | 72 | 17.5 ± 6.5 | 77.5 ± 7.4 ab | 11.3 (4.1–21.4) c | 21 (14.6–57.4) b | 0.1 ± 0.02 | – |
| – | + | 120 | 0 | 75 ± 9 ab | 10.8 (5.5–17.8) ab | 19.8 (14.4–41.9) b | 0.1 ± 0.03 | – |
| + | – | 0.017 | 22.5 ± 11,4 | 77.5 ± 11.4 ab | 9.6 (7.4–12) ab | 18 (15.2–23.2) b | 0.1 ± 0.03 | 4.3 ± 6abc |
| + | – | 12 | 27.5 ± 12 | 90 ± 8.6 b | 8.1(2.1–13.3) bc | 16.8 (12–33.7) a | 0.1 ± 0.03 | 4.4 ± 7.3bc |
| + | – | 48 | 20 ± 14.6 | 90 ± 8.6 b | 8.1 (7–9.3) ab | 12.7 (11.2–15) a | 0.3 ± 0.04 | 8.4 ± 9.6c |
| + | – | 72 | 32.5 ± 4.1 | 97.5 ± 2.2 b | 8.3 (6.5–10) ab | 15 (13–18.2) a | 0.2 ± 0.03 | 6.3 ± 7.2abc |
| + | – | 120 | 32.5 ± 7.4 | 100 b | 5.9 (5–6.7) a | 9 (8–10.4) ab | 0.4 ± 0.06 | 4.2 ± 7.9a |
| + | + | 0.017 | 27.5 ± 8.2 | 90 ± 8.6 b | 7 (5.3–8.4) ab | 13 (11–15.7) ab | 0.2 ± 0.03 | 5.5 ± 5.8bc |
| + | + | 12 | 17.5 ± 7.4 | 85 ± 10.3 ab | 8.7(7–10) ab | 15.4 (13.2–18.9) ab | 0.2 ± 0.03 | 5.8 ± 9.3abc |
| + | + | 48 | 35 ± 10.3 | 95 ± 4.3 b | 6.4 (1.8–10.2) b | 12.1 (8.7–22.1) ab | 0.2 ± 0.03 | 5.5 ± 4.8bc |
| + | + | 72 | 37.5 ± 9 | 100 b | 5.8 (5.1–6.4) a | 8.2 (7.5–9.3) a | 0.5 ± 0.06 | 7.7 ± 8.4ab |
| + | + | 120 | 38 ± 14 | 100 b | 5.2 (4.5–5.8) a | 7.8 (7–8.9) a | 0.5 ± 0.06 | 4.3 ± 6.3a |

Ten adults were exposed at 0.017 h (60 s), 12 h, 48 h, 72 h and 120 h, IP 46 conidia ($3.3 \times 10^6$ conidia/$cm^2$) formulated in KeepDry® (KD) to 0.6 mg/$cm^2$ and mineral oil Naturol® (N) 0.25 µl/$cm^2$ (IP 46 + KD + N) incubated at 75 ± 5% relative humidity and 25 ± 1 °C up to 15 days. Values (calculated with four independent replications) within the same column (mortalities with ANOVA and SNK-test, $P < 0.05$; $LT_{50}$ and $LT_{90}$ with Probit analysis on their CI 95%), followed by different letter (a–c), were significantly different. * Mortality insufficient to calculate $LT_{50}$ and $LT_{90}$. ** Values multiplied by $10^6$.

the fungus is desirable but may not be so critical as might generally be assumed. Mosquitoes diseased by an entomopathogenic fungus will survive for a couple of days but, depending on the virulence of the pathogen, feeding activity and reproduction of diseased individuals will decrease quickly after infection. Thus, the fitness and capacity to transmit pathogens and parasites are both reduced for fungus-infected mosquitoes (Blanford et al., 2005; Garza-Hernández et al., 2013; Heinig et al., 2015; Heinig and Thomas, 2015).

Fungal efficacy causing high and rapid mortality could be improved by using diatomaceous earth (KD) combined with mineral oil (N) or vegetable oil (G) at a tenfold lower conidial concentration. Additives separately, KD or N (G to a lesser extent) or in combination, KD + N and KD + G had also a clear adulticidal effect under the conditions tested here. In combination with conidia, adults were more susceptible to infection when exposed to conidia formulated with KD only or KD formulated with mineral or vegetable oil than to conidia formulated only with oil (G or N). Most studies on formulations of entomopathogenic fungi for control of mosquito adults focused on oils, whether mineral or vegetable oils (Paula et al., 2008; Mnyone et al., 2011; Carolino et al., 2014; Lobo et al., 2016). In the present study, both natural oil (N) and vegetable oil (G) were tested. Whereas vegetable oils are biodegradable and ecofriendly, a mineral oil in a fungal formulation such as N will probably increase the post-application residual effect of a mycoinsecticide due to a slower disintegration by microbial activity than for a vegetable oil. Application of mineral oils at low concentrations in a device for focal application suggests a lower harmful ambient impact than the application of oil formulations in large areas, because the device or parts can be discarded under controlled conditions. The repellency of oils to mosquito adults has been studied repeatedly, and there is so far no evidence that mosquitoes are repelled by substrates treated with oil at low concentrations under laboratory and field conditions (Mnyone et al., 2010; Lobo et al., 2016).

The enhanced activity of conidia applied in a combined oil and KD formulation, even at suboptimal conditions of humidity (RH 75%) for extra-cuticular development of the fungus, was reported for the first time in *A. aegypti* and has been shown previously only in *Triatoma infestans* (Luz et al., 2012). Whereas tests on mosquitoes were also run at 75% RH, there was no clear synergistic effect of the combined formulation in *A. aegypti* adults, as found previously with vectors of Chagas disease (Luz et al., 2012).

Adults in the present study were exposed, in the first step, continuously to the formulation. This continuous exposure will provide a consistent answer on the susceptibility of the tested insect to fungal strain and the additives and different combinations with conidia but does not really reflect the conditions that mosquitoes undergo in natural field conditions. The time of exposure to a fungus-treated substrate and the real dose of the fungal formulation deposited on the target insect´s surface during exposure are key questions to be addressed for the development of indirect application strategies of a fungal formulation. Extended and recurrent permanence of mosquitoes up to hours on a treated substrate can be expected in resting sites regardless of their sex and physiological status. Day-active *A. aegypti* adults remain in their domestic and peridomestic resting sites during the night and occasionally during the day, and a device with a treated surface simulating a resting site seems to be a first choice for focal indirect applications of a mycoinsecticide for mosquito control (Silva et al., 2018). Another useful target would be devices that simulate breeding sites where gravid females will selectively settle for oviposition, albeit for shorter periods than on more routine resting sites. In these specific devices, adults eventually get contaminated with a fungal formulation and infected by the entomopathogen (Snetselaar et al., 2014).

The efficacy of IP 46 conidia formulated or not with KD + N decreased somewhat at shorter exposure periods, tested in the second step, down to a single minute, but activity was not really hampered by the tested exposure periods. Our findings on the augmented formulations suggest possible strategies to develop new devices for the control of *A. aegypti* and other mosquito vectors. Mnyone et al. (2009) determined that a minimal 30-minute exposure of *Anopheles gambiae* adults to *M. anisopliae* or *Beauveria bassiana* conidia formulated in oil could be effective.

The recycling of the fungus on the killed host by producing a new crop of infective propagules is another key question to address, as the entomopathogen could thus be dispersed by such new conidia to other resting or breeding sites where other target insects will be infected by contact to mycotized cadavers or detached conidia in the surroundings. Entomopathogenic fungi need high moisture to emerge and to sporulate on host cadavers (Luz and Fargues, 1998), and the dispersal of the entomopathogen seems most possible by gravid females that visit aquatic breeding sites. *A. aegypti* females generally oviposit close to the water line where moisture is elevated. Infected females are naturally weakened by the pathogen and might also die there as a result of their fungal infection during or after oviposition; this would allow the fungus to emerge and to form new conidia on the dead individual that get dispersed in the breeding site. Other gravid females arriving in place for oviposition as well as their eggs and eclosing larvae may eventually get contaminated and infected with the pathogen.

*M. anisopliae* s.l. IP 46 recycled on fungus-killed mosquitoes by producing high quantities of new conidia regardless of the conidial concentrations or formulations tested. Such a conidial production on dead adults makes this strain highly interesting for applications onto new breeding sites where health agents have a difficult access for other, more standard control measures. A previous study with different strains of *B. bassiana* against *T. infestans* showed that quantitative conidiogenesis on fungus-killed individuals treated previously at different conidial concentration depended on the fungal strain (Luz et al., 1999). There is still little information about the post-mortem sporulation of entomopathogenic fungi on fungus-killed mosquitoes (Falvo et al., 2016). In the present study, the additives tested had no clear effect on quantitative conidiogenesis on cadavers probably due to the minimal amounts of the oil and diatomaceous earth remaining on the mosquito´s surface after exposure. Passive or active removal of additives on the cuticle by grooming behavior and disintegration especially of the vegetable oil probably also contributed to the low impact of these formulation ingredients on the development on dead adults.

Formulations of *M. anisopliae* s.l. IP 46 conidia with mineral oil and diatomaceous earth represent a promising tool for the development of potent strategies of focal control of this important vector with entomopathogenic fungi.

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## Conflict of interest

There are no conflicts of interest to declare.

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