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Histopathological effects of *Aspergillus clavatus* (Ascomycota: Trichocomaceae) on larvae of the southern house mosquito, *Culex quinquefasciatus* (Diptera: Culicidae)

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ARTICLE INFO

Article history:
Received 22 December 2015
Accepted 4 January 2016
Available online 14 January 2016
Corresponding Editor:
N.P. Money

Keywords:
Aspergillosis
Bioassay
Electron microscopy
Entomopathogenic fungi
Histopathology
Microbial control

ABSTRACT

Aspergillus clavatus (Ascomycota: Trichocomaceae) was previously found to be an opportunistic pathogen of mosquitoes (Diptera: Culicidae). In the present study, the mechanism leading to its insecticidal activity was investigated regarding histological damages on *Culex quinquefasciatus* larvae exposed to *A. clavatus* spores. Multiple concentration assays using spore suspensions ($0.5–2.5 \times 10^8$ spores ml^{-1}) revealed 17.0–74.3 % corrected mortalities after 48 h exposure. Heat-deactivated spores induced a lower mortality compared to nonheated spores suggesting that insecticidal effects are actively exerted. Spore-treated and untreated larvae were prepared for light microscopy as well as for scanning and transmission electron microscopy. Spores failed to adhere to the external body surface (except the mouth parts) of these aquatic immature stages but progressively filled the digestive tract where their metabolism seemed to activate. In parallel, the internal tissues of the larvae, i.e. the midgut wall, the skeletal muscles, and the cuticle-secreting epidermis, were progressively destroyed between 8 and 24 h of exposure. These observations suggest that toxins secreted by active germinating spores of *A. clavatus* in the digestive tract altered the larval tissues, leading to their necrosis

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http://dx.doi.org/10.1016/j.funbio.2016.01.002

and causing larval death. Fungal proliferation and sporulation then occurred during a saprophytic phase. A. *clavatus* enzymes or toxins responsible for these pathogenic effects need to be identified in further studies before any use of this fungus in mosquito control.

Introduction

Many mosquito (Diptera: Culicidae) species are vectors responsible for the transmission of infectious diseases of medical and veterinary importance including filariasis, malaria, and arboviruses (Goddard 2008; Mullen & Durden 2009; Medlock *et al.* 2012). Risk for human infection considerably increased during the last decades due to climate changes and increasing global trade (Reiter 2001; Medlock *et al.* 2012; Boukraa *et al.* 2013). Integrated pest management is now promoted due to harmful side effects of the chemical insecticides classically used for mosquito control and insect resistance development (Nauen 2007; Rattner 2009; Rivero *et al.* 2010). Entomopathogenic microorganisms including fungi are increasingly studied in a biological control context regarding their ability to infect and kill insect hosts with more or less selectivity (Shah & Pell 2003; Becker *et al.* 2010; Bawin *et al.* 2015). In this context, soil-borne generalist fungal pathogens including *Beauveria* (Ascomycota: Cordycipitaceae) and *Metarhizium* (Ascomycota: Clavicipitaceae) isolates have been extensively studied due to their simple life-cycle and thereby easy production of stable aerial spores which are the infectious propagules (Scholte *et al.* 2004; Kanzok & Jacobs-Lorena 2006; Seye *et al.* 2013). Despite a possible production of hazardous metabolites to vertebrates (Gugnani 2003; Bräse *et al.* 2009), *Aspergillus* (Ascomycota: Trichocomaceae) species are now studied due to the opportunistic insect pathogenic behaviour of some isolates (de Moraes *et al.* 2001; Pereira *et al.* 2009; Maketon *et al.* 2014), their ubiquity in the environment (Gugnani 2003), and their potential for biotechnological applications (Powell *et al.* 1994).

Infection of terrestrial insects as adult mosquitoes by such fungi proceeds by several steps following a consistent pattern (Shah & Pell 2003; Charnley & Collins 2007; Khachatourians & Qazi 2008), starting with spore adhesion to the host cuticle, followed by germination and penetration using enzymatic and mechanical forces. Colonization of the insect haemocoel, commonly relied with hyphal proliferation within host tissues and enzymatic activities, is responsible for lethal histopathological damages. The virulence of insect pathogenic fungi is thus related to the secretion of proteolytic, lipolytic, and chitinolytic enzymes as well as secondary metabolites (Hajek & St Leger 1994; Khachatourians & Qazi 2008). Fungal eruption through the integument of the insect finally leads to the dissemination of newly produced aerial spores. By contrast, the mechanisms by which these pathogens affect aquatic mosquito larval instars appear to be inconsistent and often unclear. Many invasion routes have been reported, including penetration of the cuticle as described above, or entry via the respiratory siphon or alimentary canal (Lacey *et al.* 1988; Miranpuri & Khachatourians 1991; Butt *et al.* 2013).

Aspergillus clavatus Desmazières (Ascomycota: Trichocomaceae) was previously investigated as an effective opportunistic mosquito pathogen (Seye & Ndiaye 2008; Seye *et al.* 2009, 2014a). However, the precise mechanism leading to larval death remains elusive. In the present study, histological damages on larvae of the southern house mosquito, *Culex quinquefasciatus* Say, during exposition to *A. clavatus* spores are described through light and electron microscopy observations.

Materials and methods

Fungal strain

A. *clavatus* was isolated from the locust cricket *Oedaleus senegalensis* Krauss (Orthoptera: Acrididae) in the Botanical Garden of the Faculty of Sciences, Cheikh Anta Diop University, Dakar, Senegal (accession number MUCL 55275, Mycothèque de l'Université Catholique de Louvain, Belgian Coordinated Collections of Microorganisms, Belgium) and maintained on Potato Dextrose Agar (PDA) medium as pure culture. This strain was previously shown to have an insecticidal activity against mosquitoes (Seye *et al.* 2009).

To obtain large amounts of spores, the fungus was grown in 250 ml Erlenmeyer flasks on a wheat bran-based solid-state substrate (5 g wheat bran per flask and 20 ml nutritive solution: peptone 1 %, yeast extract 1 %, chloramphenicol 0.005 %). Wheat bran and nutritive solution were separately sterilized at 121 °C for 20 min before being mixed. Each flask was inoculated with a spore suspension (1 ml, 5×10^6 spores ml^{-1}) and incubated 7 d at 30 °C. Fungal masses produced on the media were then washed with a 0.05 % Tween 80 solution in distilled water (150 ml) on a rotary shaker (150 rpm) for 2 h. Fungal filaments and wheat bran residues were discarded by filtration through a double layer of sterile muslin, and the resulting solution was centrifuged (3000 g, 5 min) to remove the newly produced spores as pellet. Spores were resuspended in a 0.05 % Tween 80 solution and the concentration was assessed using a haemocytometer (Thoma, Assistent, Sondheim/Rhön, Germany) before application on mosquito larvae.

Mosquito larvae

C. *quinquefasciatus* adults (S-lab strain, native from Riverside, California) were reared in 50 × 50 × 50 cm net cages (BugDorm, MegaView Science, Taichung, Taiwan) and fed with 10 % (w/v) sucrose solutions in water. Females were exposed three times a week to glass membrane feeders (manufactured by CNAP-MAD, Antananarivo, Madagascar) maintained at 37 °C by circulating water, to allow blood meal through stretched Parafilm sheets. Plastic cups filled of water were placed in

the cages as oviposition sites. Egg rafts were daily collected and maintained in 25 (length) × 15 (width) cm containers with 5 (depth) cm distilled water. After hatching, larvae were fed every 2 d with a powder made with 2:1 crushed fish food (TetraMin, Tetra, Blacksburg, USA) and natural brewer's yeast (Biover, Brugge, Belgium). Water was renewed every week. Rearing conditions were 25 ± 2 °C temperature, 70 ± 5 % relative humidity, and 16:8 h (light:dark) photoperiod.

Bioassays

Conventional toxicity tests were first conducted in aqueous suspensions according to the World Health Organization protocol (2005) to assess lethal concentrations of *A. clavatus* spores required to kill 50 % (LC_{50}) and 90 % (LC_{90}) of the larvae. Batches of 20 third-instar individuals were exposed to a range of final concentrations of 0.5×10^8, 1.0×10^8, 1.5×10^8, 2.0×10^8, and 2.5×10^8 spores ml^{-1} in separated bottles (50 ml) for 48 h. Control larvae were maintained in 0.05 % Tween 80 solution. Treatments (i.e. a control batch of larvae associated to a set of the tested concentrations) were replicated four times at different time intervals with spores produced independently. Laboratory conditions were similar to rearing conditions. Mortality was daily recorded and corrected using the Abbott's formula (Abbott 1925). Corrected mortality proportions were linearized using logit transformation (Dagnelie 1970): $\text{logit}(P) = \ln(P/1 - P)$. A simple linear regression was used for modelling the relationship between logit-transformed mortality and logarithm-transformed values of fungal concentrations as explanatory variable: $\text{logit}(P) = \text{slope} \times \ln(\text{concentration}) + \text{intercept}$. The relationship between larval mortality and spore concentrations was assessed considering Snedecor-*F* distribution and *p*-value.

The lethal activity of the fungus was then investigated over time. Toxicity tests were carried out as described above using a concentration of 1.5×10^8 spores ml^{-1} (that is related to LC_{50}s). Mortality was recorded after 6, 12, 18, 24, 30, 36, and 48 h exposure. The lethal time required to kill 50 % of the larvae (LT_{50}) was calculated using Kaplan–Meier analysis, and log-rank test (providing chi-square and *p*-value) was carried out to check for significant differences in survival between spore-treated and control larvae.

Heat-treated spores were used to determine whether spore germination is required to impact mosquito larvae. Spores in aqueous suspension were killed by autoclaving for 20 min at 121 °C. Spore viability was assessed using PDA plates over an incubation time of 72 h at 30 °C. Toxicity tests were conducted as described above using a 2.5×10^8 spores ml^{-1} concentration. Differences in survival between individuals exposed to heat-treated and nonheated spores, and control larvae were analysed using Kaplan–Meier analysis with pairwise comparison over log-rank test.

Dead larvae treated with either 2.5×10^8 intact or heat-deactivated spores ml^{-1} ($n = 10$ per treatment) were randomly sampled, rinsed thrice with distilled water, and incubated on a wet paper in Petri dishes (30 °C) in order to observe the emergence of fungal aerial filaments outside the body.

All statistical analyses were performed using Minitab v.17 software (Minitab, Coventry, UK). For all tests, the significant threshold was $p < 0.050$.

Light and electron microscopy

Fifty third-instar larvae (125 ml) were exposed to a concentration of 1.5×10^8 spores ml^{-1} according to the protocol described above. Control larvae were maintained in 0.05 % Tween 80 solution. In both treatments, five larvae were taken after 8, 12, 24, and 48 h exposure for light microscopy (LM) and transmission electron microscopy (TEM). Three additional larvae were sampled after 8 and 24 h for scanning electron microscopy (SEM).

Spore-treated and control larvae were cut behind the head and in front of the respiratory siphon to facilitate the permeation of chemicals. Body segments were fixed for 12 h in a 2.5 % glutaraldehyde solution buffered with 0.1 M sodium cacodylate at pH 7.3. They were then rinsed and stored for few days in 0.2 M cacodylate buffer, before postfixation in 1 % osmium tetroxide (1 h), and three rinses (3 × 10 min) in distilled water. Dehydration was performed through a graded ethanol series of increasing concentrations: 30 %, 50 %, 70 %, 90 % (1 × 10 min), and 100 % (3 × 20 min).

For LM and TEM, body segments were embedded in epoxy resin (SPI-PON 812, SPI-CHEM, SPI supplies, Leuven, Belgium) with propylene oxide as intermediate solvent for impregnation (2 × 30 min in pure solvent, 2 h 30 min in solvent/resin mixture, and overnight in pure resin). Embedding was performed in flat silicone molds to facilitate sample orientation for sectioning, then placed in a stove (60 °C, 72 h) to allow polymerization. Semithin (1–2 μm) and ultrathin (60–80 nm) sections were performed between the second and fourth abdominal segment using a diamond knife on a Reichert-Jung Ultracut E (Reichert-Jung, Vienna, Austria) ultramicrotome. Semithin sections were stained with 1 % toluidine blue (pH 9) before observation for general histology and orientation of further ultrathin sections, using an Olympus Provis Ax70 (Olympus, Tokyo, Japan) light microscope equipped with an Olympus DP50-CU (Olympus) digital camera. Ultrathin sections were contrasted with uranyl acetate and lead citrate according to the conventional method; then observed in a Jeol JEM 100-SX (JEOL, Tokyo, Japan) transmission electron microscope under 80 kV accelerating voltage.

For SEM, dehydrated body segments were critical-point dried with CO_2 and mounted on glass slides with double-side carbon tape. All were then sputter-coated with Pt (20 nm) in a Blazers SCD 030 unit (Oerlikon Balzers Coating, Balzers, Liechtenstein) before observation in a Jeol JSM-840A (JEOL, Tokyo, Japan) scanning electron microscope under 20 kV accelerating voltage.

Results

Bioassays

Control mortalities ranged from 0.0 (24 h) to 2.9 % (48 h). Corrected mortalities increased with the spore concentration ($0.5–2.5 \times 10^8$ spores ml^{-1}), ranging from 12.5 to 67.5 % at 24 h and from 17.0 to 74.3 % at 48 h. Corresponding lethal concentrations (LC_{50} and LC_{90}) were respectively 1.4×10^8 and 4.9×10^8 spores ml^{-1} (slope = 1.8 ± 0.3; intercept = −33.5 ± 4.9;

$F_{(1,18)} = 45.8$; $p < 0.001$), and 1.2×10^8 and 4.3×10^8 spores ml^{-1} (slope $= 1.7 \pm 0.2$; intercept $= -31.4 \pm 4.1$; $F_{(1,18)} = 58.1$; $p < 0.001$).

At a concentration of 1.5×10^8 spores ml^{-1}, the LT_{50} was 30.2 ± 1.9 h and survival significantly differed from control larvae ($\chi^2_{(0.95;\ 1)} = 65.8$; $p < 0.001$) (Fig 1). Natural movements of the dying larvae were impaired in the early observation times, these being unable to normally stand behind the water surface.

No A. *clavatus* spore germinated over a 72 h incubation time on PDA plates after being heat-treated. Mortality in larvae exposed to heat-treated spores (ranging from 10.0 to 19.5 % after 24 and 48 h exposure with 2.5×10^8 spores ml^{-1}) was significantly lower than larvae exposed to same amounts of non-heated spores ($\chi^2_{(0.95;\ 1)} = 30.3$; $p < 0.001$), but higher than control larvae ($\chi^2_{(0.95;\ 1)} = 4.3$; $p = 0.038$).

Light and electron microscopy

The gross morphology of C. *quinquefasciatus* larvae is shown in Fig 2A. SEM observations did not reveal spores adhering to the cuticle of the larval body, nor to the siphon. Only few of them were found on the external cuticle while they were numerous on the setae around the mouth from 8 h exposure (Fig 2B and C). Semithin sections revealed a progressive accumulation of spores between 8 and 24 h in the larval digestive tract (Fig 3C) compared to control larvae (Fig 3A and B). This accumulation occurred with a gradual destruction of the digestive epithelium (midgut), the muscles and connective tissues, and finally the epidermis below the cuticle. After 48 h, larvae appeared almost devoid of internal organs or tissues, except the peritrophic membrane enclosing spores (Fig 3D).

Spores in larval gut

Spore cell structures were indistinct at 8 h exposure, then became more obvious over time (Fig 4A and B). Since the 12 h sampling time, spores swelled and showed evidence of intracellular organelles (nuclei, mitochondria) and membrane folds.

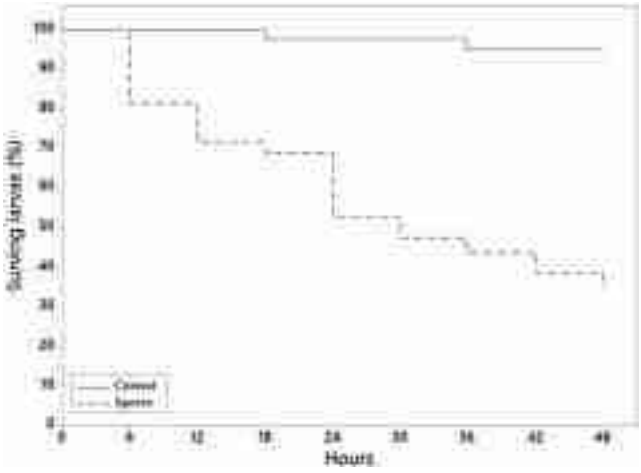

Fig 1 – Kaplan–Meier survival curve for *C. quinquefasciatus* larvae (third instar) exposed to *A. clavatus* spores (1.5×108 spores ml^{-1}) for 48 h (four replicates).

Larval gut

The first alterations observed in spore-exposed larvae (Figs 3C and 5B) compared to control larvae (Figs 3B and 5A) were the loss of microvilli at the midgut cell apex since 8 h and the loss of adhesion between adjacent cells since 12 h. Moreover, deeply altered intracellular organelles gave rise to vacuoles in the cytoplasm. The midgut epithelium appeared disrupted in many locations within 24 h (Fig 3C) with some cells being completely destroyed; whereas others showed breaks of apical cellular contacts and/or were detached from the basal membrane. The peritrophic membrane remained however intact and still enclosed the bolus regardless of the duration of the treatment (Fig 3C and D).

Larval muscles

TEM views also revealed that muscles were gradually disorganized over the treatment time in spore-treated larvae (Fig 6B and C) compared to control larvae (Fig 6A). The sarcoplasmic reticulum was disturbed after 8 h exposure, deeply vacuolized after 12 h, and almost absent in the sections after 24 h. This loss occurred with a dispersion and alteration of myofibrils that were reduced in size and number of filaments.

Cuticle-secreting epithelium

The cuticle-secreting epithelium of spore-treated larvae (Fig 7B) also underwent alterations when compared to control larvae (Fig 7A). The disappearance of cell organelles as mitochondria and Golgi apparatus was first observed after 8 h exposure. No organelles were recognized after 24 h and large vacuoles filled most of the cell volume. The cuticle however still remained in place.

Saprophytic phase

The midgut wall was disrupted within 24 h and larvae died. At this time, ingested bacilliform bacteria (Fig 2C) increased in number in the bolus (Fig 8A) and progressively colonized the surrounding tissues. After 48 h, the larval haemocoel was invaded by bacteria (Fig 8B) and almost devoid of internal organs (Fig 3D).

Further macroscopic observations revealed that aerial fungal filaments emerged outside the larval body (Fig 8C) after 48 h incubation in Petri dishes (thus 96 h after treatment). None was observed in the case of larvae treated with heat-deactivated spores.

Discussion

The present study confirms the insecticidal potential of A. *clavatus* spores against C. *quinquefasciatus* larvae as previously reported (Seye *et al.* 2009, 2014a), showing a concentration–mortality relationship. The almost absence of spores adhering to the outside cuticle (except on mouthpart setae), and the lack of cuticle alteration and/or perforation visible in light and electron microscopy until 48 h exposure, lead to reject the hypothesis of spore effect from outside the larvae. Our results rather suggest that the observed mortality came from the activity of ingested spores that accumulated and filled the digestive tract.

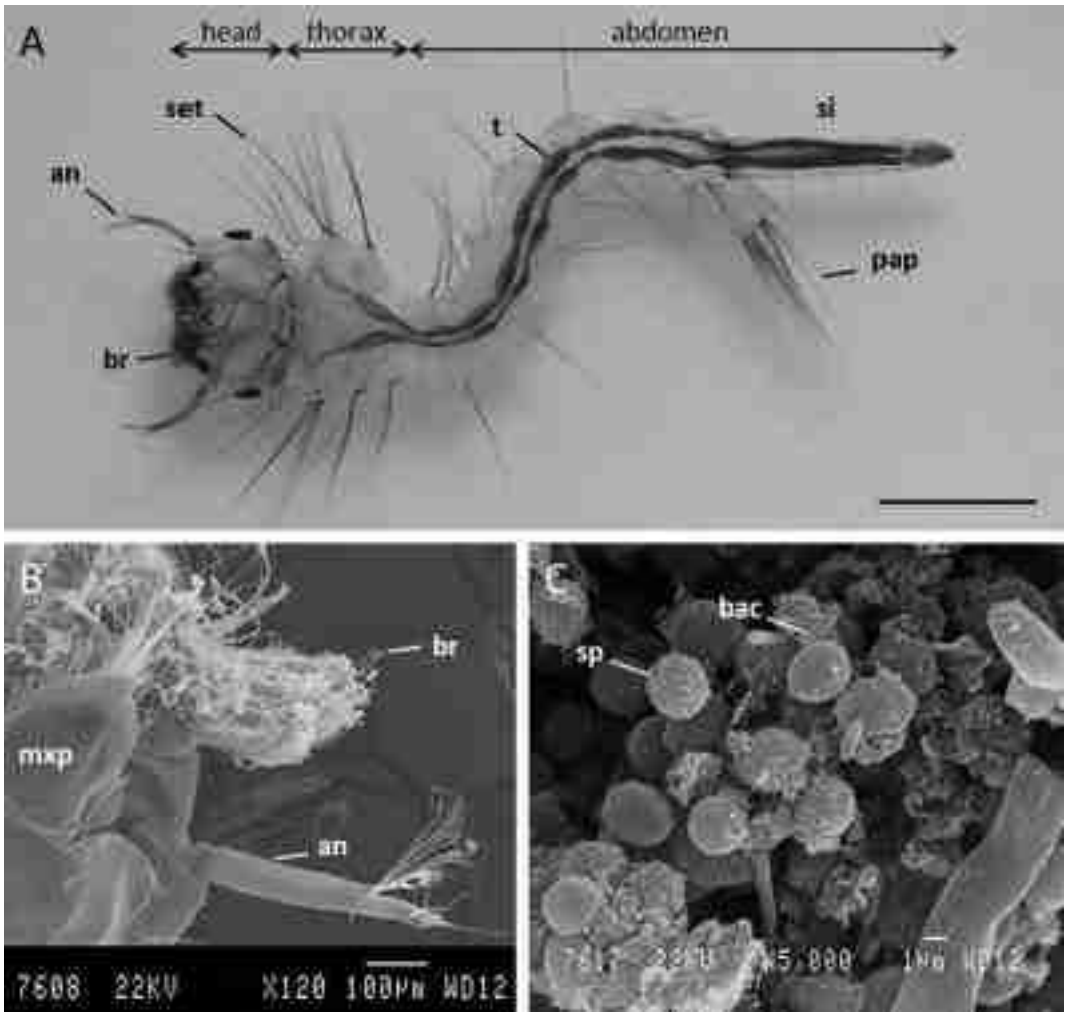

Fig 2 – (A) Gross morphology (dorsal view) of a healthy *C. quinquefasciatus* larva (scale bar = 1 mm). The entire body (head, thorax, and abdomen) is covered with setae (set). Antennae (an), maxillary palps (mxp), and lateral brushes (br) on the head enclose the mouth. Tracheal trunks (t) surrounding the digestive tract are visible through the cuticle. The abdomen is ended with a respiratory siphon (si) and two pairs of lucent anal papillae (pap) surrounding the anus. (B) SEM microphotograph (ventral view) of the head of a *C. quinquefasciatus* larva treated with *A. clavatus* spores (8 h exposure), showing the propagules adhering to the lateral brushes. (C) SEM microphotograph of *A. clavatus* spores (sp) adhering to the lateral brushes of a *C. quinquefasciatus* larva. bac, bacteria.

One concern about the absence of spore adhesion to the larval cuticle is that attachment may have been impaired by the protocol. An impact of the application method on spore invasion route was previously observed on *C. quinquefasciatus* larvae subjected to *Metarhizium anisopliae* (Metchnikoff) Sorokin (Lacey *et al.* 1988). In that study, dry hydrophobic spores covering the water surface invaded the respiratory siphon of larvae, causing their death by suffocation. By contrast, detergent-wetted spores were ingested by larvae and progressively filled their digestive tract. Death followed within 24 h due to toxins released during both early germination of the spores or partial digestion of these ones. Indeed, Bukhari *et al.* (2010, 2011) emphasized that surfactants intended to overcome the hydrophobic nature of fungal spores and obtain homogeneous aqueous suspensions would rather inhibit spore adhesion on mosquito larvae cuticle. However, another study conducted with *Aedes aegypti* Linnaeus larvae treated with spore suspensions of *Beauveria bassiana* (Bals.-Criv.) Vuillemin revealed that spores adhered to the external cuticle (particularly the mouth parts and the anal region) and lead to the larval death (Miranpuri & Khachatourians 1991). Switching invasion route due to the protocol cannot thus be raised as an empirical rule. It should also be noted that the *Aspergillus* strain used in this study is a soil-borne opportunistic pathogen. Considering terrestrial insects, spores are known to attach to the cuticle via passive hydrophobic forces and subsequent secretion of anchoring compounds (Khachatourians & Qazi 2008). Some *Aspergillus* species including *A. clavatus* were found to infect such organisms according to this scheme (Kumar *et al.* 2004; Seye *et al.* 2014b). Therefore, the lack of spore adhesion may also be due either (1) to the dilution in water of the mucilaginous

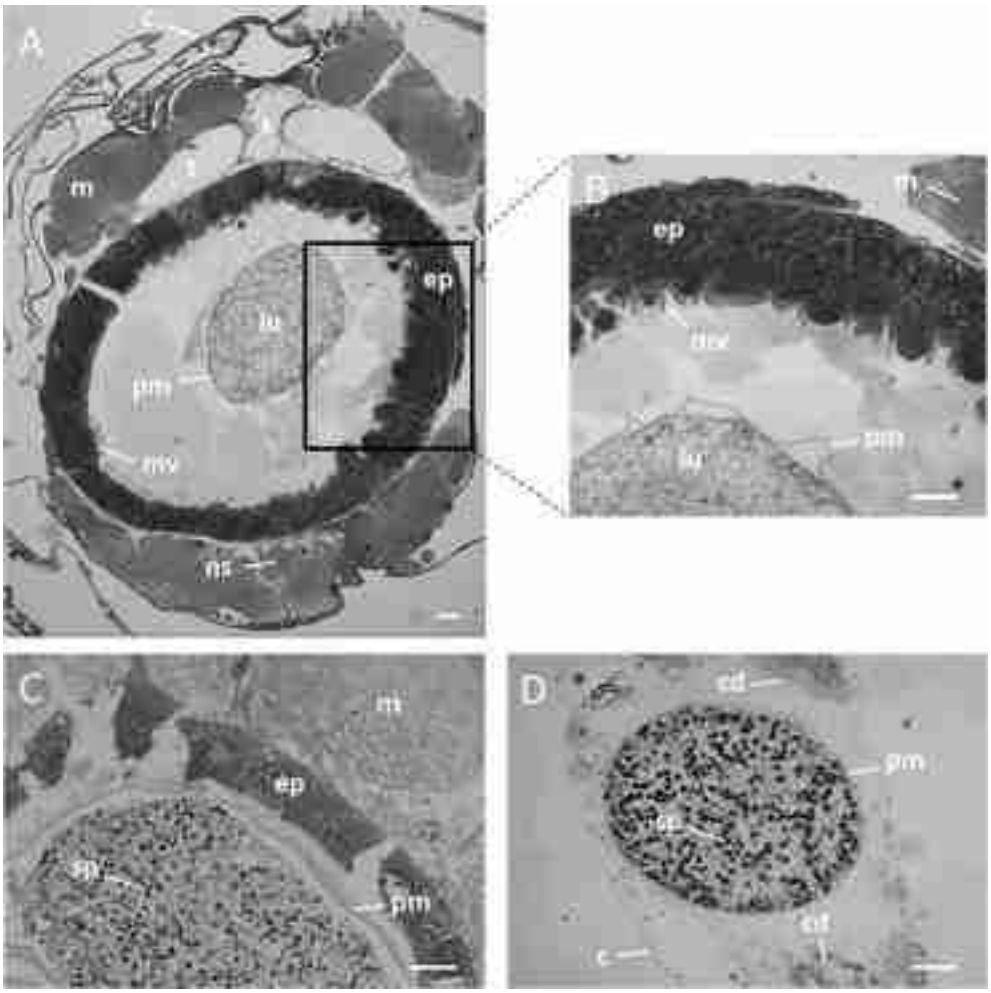

Fig 3 – Semithin cross-sections of control (A, B) and spore-treated (C = 24 h, D = 48 h) larvae of *C. quinquefasciatus* showing a portion of skeletal muscles (m) and digestive tract (midgut) with the bolus surrounded by a peritrophic membrane (pm). In control larvae, the muscles appeared as compact fibres, the columnar midgut epithelium (ep) was continuous and displayed an evident brush border of microvilli (mv). In spore-treated larvae, the number of spores (sp) increased in the bolus and tissues were progressively disorganized. Scale bar = 20 μm. c, external cuticle; cd, cellular debris; lu, digestive tract lumen; ns, ventral nervous system; t, tracheal trunk; s, dorsal blood sinus.

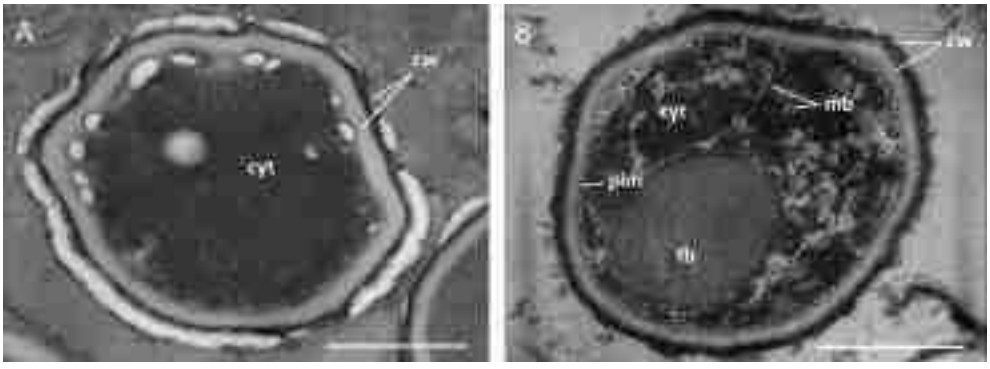

Fig 4 – TEM microphotographs of *A. clavatus* spores in the digestive tract of *C. quinquefasciatus* after 8 h (A) and 48 h (B) exposure. Scale bar = 1 μm. cw, cell wall; cyt, cytoplasm; fb, fat body; mb, membrane fold; plm, plasma membrane.

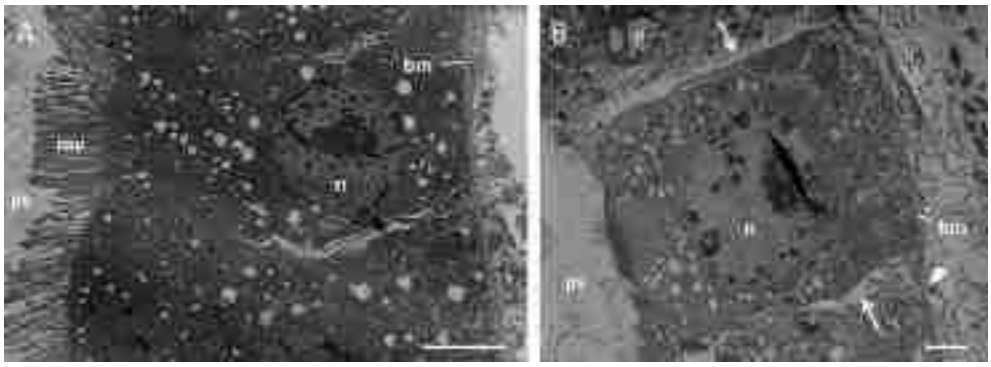

Fig 5 – TEM microphotographs of digestive epithelial cells in control (A) and spore-treated (B = 24 h) larvae of *C. quinquefasciatus*. In control larvae, the intracellular organelles were obvious and numerous microvilli (mv) covered the apical cell surface in contact with the peritrophic space (ps). In spore-treated larvae, microvilli were detached and cell contacts disrupted (arrows) while some vacuoles (v) appeared in the cytoplasm. Scale bar = 5 μm. bm, basal membrane; n, nucleus.

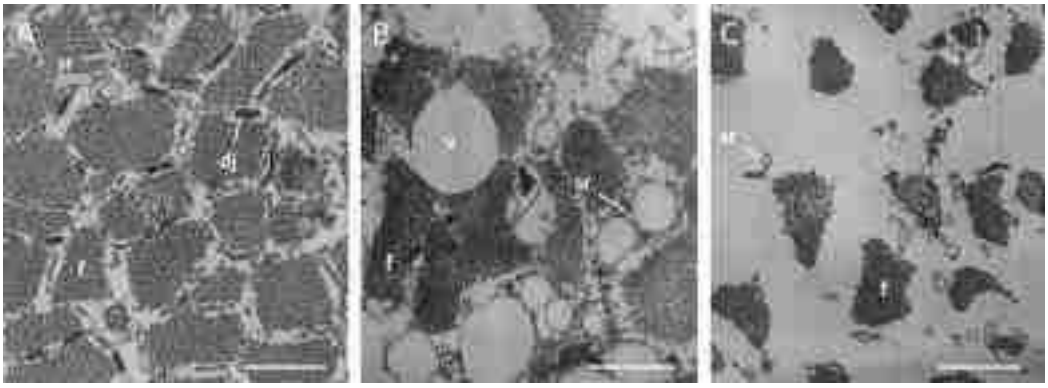

Fig 6 – TEM microphotographs of cross-sectioned skeletal (striated) muscle fibrils in control (A) and spore-treated (B = 12 h, C = 24 h) *C. quinquefasciatus* larvae. In control larvae, well organized myofibrils (f) with densely distributed myofilaments (actin and myosin) are surrounded by a sarcoplasmic reticulum (sr). In spore-treated larvae, the sarcoplasmic reticulum is vacuolized within 12 h (B) and almost disappeared within 24 h (C), while broadly spaced fibrils subsisted and were reduced in sizes. Scale bar = 1 μm. dj, diad junction; v, vacuole.

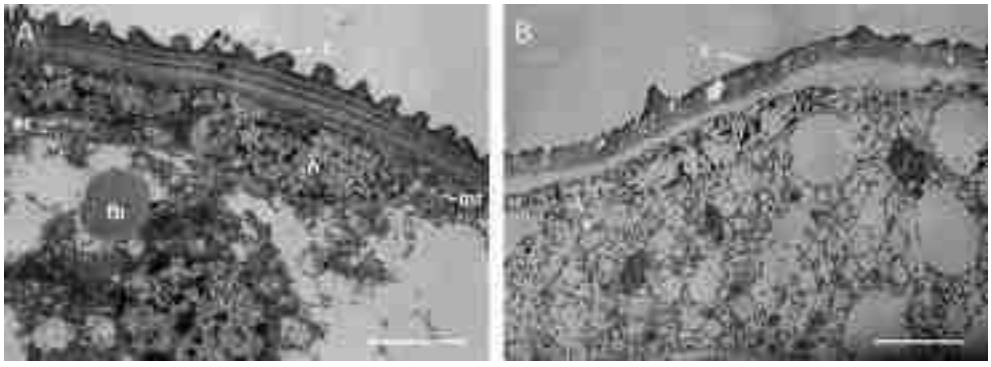

Fig 7 – The usual organization of cuticle-secreting epidermis in control larvae of *C. quinquefasciatus* (A) was shown to be disturbed in spore-treated larvae (B = 24 h) by the disappearance of organelles and the almost complete vacuolization of the cells. Scale bar = 5 μm. c, cuticle; er, endoplasmic reticulum; fb, fat body; mt, mitochondria; n, nucleus; v, vacuole.

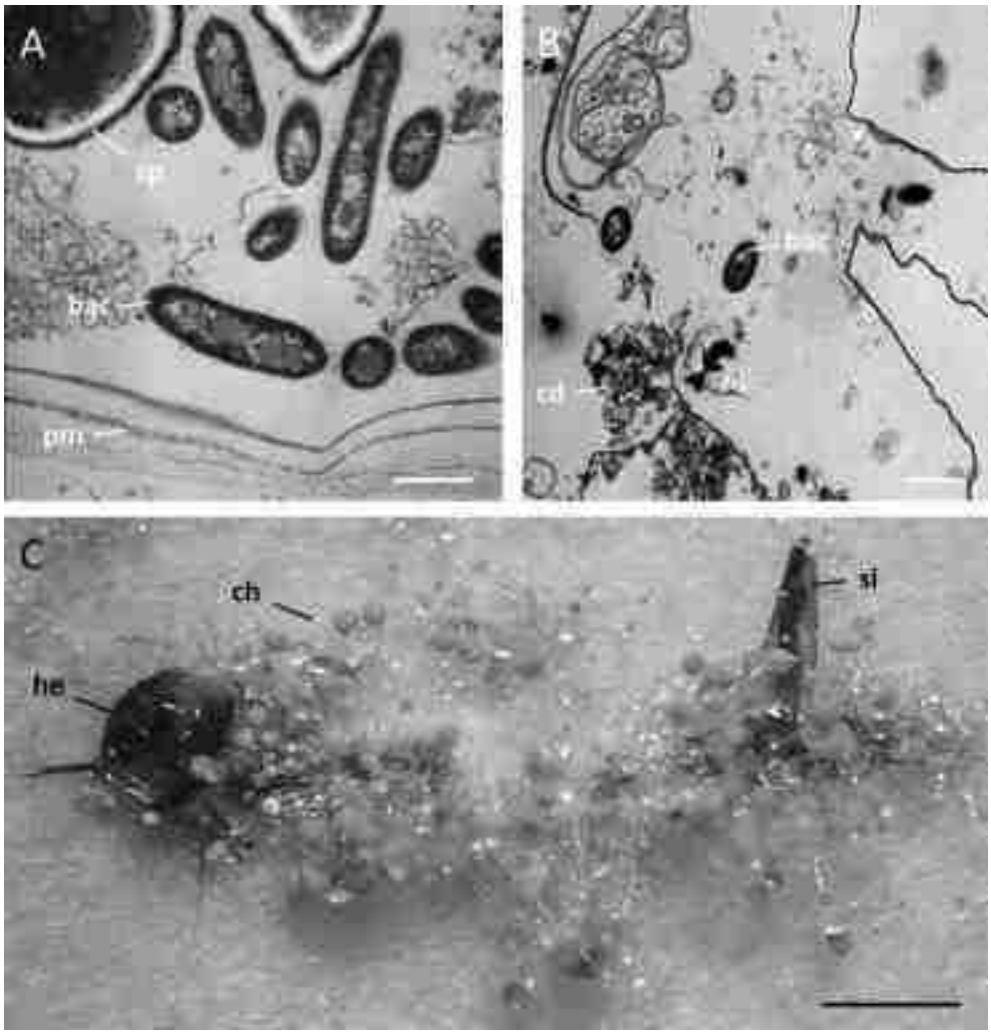

Fig 8 – (A) Ingested bacilliform bacteria (bac), associated with spores (sp) in the bolus that is surrounded by the peritrophic membrane (pm), increased in number since 24 h after treatment (scale bar = 1 μm). (B) Some invaded the larval haemocoel and were found nearby cellular debris (cd) (scale bar = 1 μm). (C) C. *quinquefasciatus* larva incubated in Petri dish 48 h after death, displaying *A. clavatus* aerial filaments that emerged outside the body (scale bar = 1 mm). he, head; ch, conidial head; si, siphon.

compounds produced by the fungus to attach the cuticle (Butt *et al.* 2013), or (2) to the physico-chemical cues of the cuticle with some compounds protecting the insect against potential microbial pathogens by altering hydrophobicity (Lord & Howard 2004) or being fungistatic (Koidsumi 1957; Sosa-Gomez *et al.*, 1997; Urbanek *et al.* 2012; Golebiowski *et al.* 2015). It was also hypothesized that the gut of aquatic insects may be the preferred site for fungal spore development due to physiologically favourable features (Miranpuri & Khachatourians 1991), suggesting stress from the water environment. Under these assumptions, spore adhesion previously reported on mosquito larvae with *A. clavatus* by Seye *et al.* (2009) appears to be a minor epiphenomenon responsible for a limited part of the observed mortality compared to ingestion. By contrast, mosquitocidal aquatic pathogens with better spore water affinity such as *Lagenidium giganteum* Couch (Oomycota: Pythiaceae) (Kerwin 2007) or *Tolypocladium cylindrosporum* Gams (Ascomycota: Ophiocordycipitaceae) (Soares 1982) were found to adhere and penetrate the larval cuticle as reported with terrestrial insects. Whether naturally mosquito-associated *Aspergillus* isolates (de Moraes *et al.* 2001; Pereira *et al.* 2009; Mohanty & Prakash 2010) are more adapted to aquatic environments or simply ingested by larvae as described above before being isolated remains to be investigated.

Whether ingested spores mechanically blocked the mouth parts while others attached inside the digestive tract (Federici 1981) or not (Butt *et al.* 2013) has still to be elucidated. Anyway, they remained enclosed in the peritrophic membrane that was apparently not affected. This suggests that damages to larval tissues were caused at distance by an early diffusion of toxins or enzymes. On the one hand, dead larvae were reported after exposition to heat-deactivated spores, and one might expect that at least partial digestion of spores and/or passive diffusion of toxins occurred being responsible for a few part of the mortality. However, living spores were

required to fully bring about larval mortality. As revealed by TEM observations, damages to larval tissues may indeed be related to the appearance of ultrastructural features of spore metabolic activity. Considering previous reports, Lacey *et al.* (1988) observed the early germination steps of *M. anisopliae* spores in the digestive tract of dying larvae suggesting that death arose due to secreted toxins. Miranpuri & Khachatourians (1991) have established the kinetics of *B. bassiana* spore development in the digestive tract of *Ae. aegypti* larvae. An ingested spore (stage I) swells twice its size in 24 h (stage II). A germ tube (stage III) then emerges up to 48 h later, followed by linear (stage IV) and branched (stage V) mycelial growth. These observations are consistent with the results presented here with respect to the first two stages. Extended observation times would allow to show the emergence of a germ tube, as incubated dead larvae displayed aerial fungal filaments. On the other hand, the ultrastructural damages observed in larval tissues are characteristic of cell lysis and tissue necrosis. Especially, the serious damages affecting the epithelial midgut cells are similar to that caused by *Bacillus thuringiensis* var. *israelensis* Berliner endotoxins against *Simulium pertinax* Kollar (Cavados *et al.* 2004) and *Aedes albopictus* Skuse larvae (Silva *et al.* 2008), as well as aqueous plant extracts against *Anopheles gambiae* Giles (Koua *et al.* 1998) and *C. quinquefasciatus* (Almehmadi, 2011), where loss of microvilli, vacuolization of digestive cells and rupture of the epithelium, and rejection of cytoplasmic material in the peritrophic space were also described. Taking together, these observations (spore activity and characteristic tissue necrosis) are consistent with the hypothesis where spores actively released during germination toxic compounds that are responsible for cellular damages. The degradation of the larval tissues may then have been reinforced by ingested saprophytic bacteria that benefited from the knockdown of the immune system and invaded the general cavity in the last steps.

A. clavatus is a commonly encountered fungus in the environment known to produce a large number of enzymes and mycotoxins (Gugnani 2003; Bräse *et al.* 2009). Many studies showed that *A. clavatus* germination was accompanied by a secretion of toxins such as clavatol (Bergel *et al.* 1944), ascladiol (Suzuki *et al.* 1971), tryptoquivalone and tryptoquivaline (Clardy *et al.* 1975), glyantrypine (Penn *et al.* 1992), cytochalasin E (Lopez-Diaz & Flannigan 1997), kojic acid and xanthocilin (Pitt 2000), and patulin (Sabater-Vilar *et al.* 2004). Some of these compounds acting synergistically are responsible for human diseases and animal poisoning (Flannigan & Pearce 1994; Lopez-Diaz & Flannigan 1997). Whether the observed tissue disorders are due to such nonselective compounds remain to be determined as these features depend on the fungal isolate. By example, patulin production was detected in three of eight tested *A. clavatus* strains (Varga *et al.* 2003), with amounts being strain dependent (Lopez-Diaz & Flannigan 1997). However, the fact that the chitinous peritrophic membrane as well as the external cuticle remained intact until at least 48 h exposure suggests that early germinating spores did not secrete adapted chitinolytic nor proteolytic enzymes, contrary to that was reported with other insect pathogenic fungi (Schrank & Vainstein 2010). Because heat-denatured spores induced some larval mortality, heat-stable compounds such as mycotoxins may be at least in part responsible for the pathogenic effects. Patulin, which was found to slowly decompose at 90 °C while over 20 % of the compound only disappeared in 30 min at 120 °C (Kubacki 1986), can be one of such expected toxins. Also, cytochalasin E produced by *A. clavatus* was reported to induce an inhibition of glucose transport and actin polymerization (Brenner & Korn 1980). This could explain why natural movements started to stop down in the early observation times. At this time, some larvae were no more able to breathe air on the water surface probably causing their asphyxia.

Conclusions

Our results suggest that toxic compounds caused damages to larval tissues through ingestion and accumulation of spores in the digestive tract, which would therefore appear as the primary invasion route. Living spores are required to fully bring about larval mortality, suggesting that these compounds are actively secreted. Their nature and properties are thus of great concern before using *A. clavatus* spores in environmental conditions due to a likely lack of specificity and maybe side effects on nontargeted organisms. Further studies dealing with the purification of *A. clavatus* toxins are required in order to characterize the compounds responsible for the lethal effects.

Acknowledgements

We thank the Islamic Development Bank (IDB) for the financial support of a postdoctoral scholarship to Fawrou Seye. Nicole Decloux (Functional Morphology and Evolutionary, University of Liege) is acknowledged for her technical assistance in the ultrastructural study and the Center of Applied Technology in Microscopy (CATμ, University of Liege) for providing access to electron microscopy equipment.

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Appl Entomol Zool (2014) 49:453–458
DOI 10.1007/s13355-014-0273-z

ORIGINAL RESEARCH PAPER

Effect of entomopathogenic *Aspergillus* strains against the pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae)

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Received: 27 December 2013 / Accepted: 11 May 2014 / Published online: 6 June 2014

Abstract Aphids (Homoptera: Aphididae) are sap-sucking insect pests that feed on several plants of agronomical importance. Entomopathogenic fungi are valuable tools for potential aphid control. As part of a selection process, laboratory bioassays were carried with five different concentrations of *Aspergillus clavatus* (Desmazières), *Aspergillus flavus* (Link) and *Metarhizium anisopliae* ((Metschnikoff) Sorokin) spores against the pea aphid, *Acyrthosiphon pisum* (Harris). *Aspergillus* isolates induced higher mortalities than *M. anisopliae*, which is a well-known entomopathogen in the literature. Lethal concentrations (LC_{50} and LC_{90}) were 1.23×10^3 and 1.34×10^7 spores/ml for *A. flavus*, 4.95×10^2 and 5.65×10^7 spores/ml for *A. clavatus*, and 3.67×10^3 and 9.71×10^7 spores/ml for *M. anisopliae* 5 days after treatment. Mycelia development and sporulation on adult cadavers were observed 48 h after incubation. The intrinsic growth rate of *A. pisum* decreased with increased spore concentration for all fungal strains, suggesting an increase in pathogen fitness related to a consumption of host resources. In conclusion, *Aspergillus* species could be useful in aphid control as pest control agents despite their saprophytic lifestyle. This is also to our knowledge the first report of *A. clavatus* and *A. flavus* strains pathogenic to aphids.

Keywords Aphid · *Aspergillus* · *Metarhizium* · Entomopathogenic fungi · Biological control

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Introduction

The *Acyrthosiphon pisum* (Harris) aphid (Homoptera: Aphididae), also called pea aphid, is a pest of many cultivated and wild plants such as the worldwide crop *Vicia faba* (L.), limiting their growth. These aphids have also been reported to be a vector of some viral diseases (Brault et al. 2010; Emden and Harrington 2007). Different strategies have been employed to control these pests. The most widely used methods are physical, chemical and more recently an integrated approach that includes biological control using many kinds of organisms as parasitoids and pathogens. Entomopathogenic fungi are well known to be effective against insects. Their pathogenicity depends on the fungal species and strain virulence. In the latter case, substrate and culture methods are of primary

importance for spore effectiveness by increasing or decreasing the secretion of insecticidal compounds recognized as virulence factors (Shah et al. 2005). In this context, agricultural by-products could be used as inexpensive media with high spore yield production (Sahayaraj and Namasivayam 2008). Various entomopathogenic fungi such as *Lecanicillium lecanii* (Zare and Gams) (Jung et al. 2006), *Beauveria bassiana* ((Balsamo) Vuillemin) (Sivasundaram et al. 2007) and *Metarhizium anisopliae* ((Metschnikoff) Sorokin) (Dong et al. 2007) have been effectively used to control aphids, lepidopteran larvae and other pests. Vu et al. (2007) also demonstrated the pathogenicity of many entomopathogenic fungi such as *M. anisopliae* against aphids. Many other studies have shown the pathogenicity of fungi against aphids (Hall 1979, 1980; Khan et al. 1990), but by direct application on the cuticle or by leaf treatment. However, because of the high growth rates of aphid populations, it is unlikely that they can be fully controlled in all cases. Due to emerging insecticide resistances, the research into biological control of aphids still continues. In this present work, we showed the effectiveness of three fungal isolates, *M. anisopliae*, *Aspergillus flavus* (Link) and *A. clavatus* (Desmazières), regarding the adult mortality and population growth rate by directly treating *V. faba* plants infected with *A. pisum* aphids in laboratory conditions.

Materials and methods

Fungal cultures

All the fungal strains were identified by the Mycothèque de l'Université catholique de Louvain (MUCL, Belgium). *M. anisopliae* (Seye et al. 2012, 2013) and *A. clavatus* (Seye and Ndiaye 2008; Seye et al. 2009; accession no. MUCL 55275) were recently isolated from *Oedaleus senegalensis* (Krauss) (Orthoptera: Acrididae). *A. flavus* (accession no. MUCL 55276) was isolated from *Agriotes lineatus* (L.) larvae (Coleoptera: Elateridae). All these isolates were cultured in 250-ml Erlenmeyer flasks on a solid-state substrate constituted by 5 g wheat bran supplemented with 20 ml of a nutrient solution (1 % peptone, 1 % yeast extract, 0.005 % chloramphenicol) and previously sterilized at 121 °C for 20 min. Seven days after, fungal masses produced on the media were washed with 150 ml distilled water containing 0.05 % Tween 80 on a rotary shaker (150 rpm) for 2 h. These solutions were then filtered and centrifuged (8000 rpm, 15 min) to remove the conidia. For each strain, the conidia were diluted in sterilized water containing 0.05 % Tween 80, and final suspensions were adjusted to 10^3, 10^4, 10^5, 10^6 and 10^7 spores per ml using a hemocytometer (Thoma®).

Aphid rearing

A. pisum aphids were isolated on *Phaeolus vulgaris* L. (France, 2011) and reared in laboratory conditions on ordinary broad bean (*V. faba*) plants growing in perlite/vermiculite mix pots. Rearing conditions were a 16L:8D photoperiod, 75 % relative humidity and 25 ± 2 °C temperature.

Bioassays

For each conidial suspension, 20 adult female aphids were dropped on a young plant in a pot (10 cm diameter) surrounded by a transparent plastic sleeve closed at the ends with muslin and directly treated. Each fungal strain was applied (1.6 ml of conidial suspension) with a dedicated sprayer (Di Martino spa—Conico 1000) to 20 cm with the top of the plant. This volume was sufficient to cover the entire system without impacting aphid viability. The control aphids were treated only with distilled water containing 0.05 % Tween 80. Bioassay conditions were a 16L:8D photoperiod, 75 % relative humidity and 25 ± 2 °C temperature. Aphid mortality and nymphs produced were recorded daily for 5 days after inoculation. All newborn nymphs and dead adults were removed. The cadavers were transferred to plates with moist filter paper in order to allow fungal filament emergence and placed in room conditions as previously described. Each treatment was made in four replicates of 20 adult female aphids with a new conidial suspension in different time periods.

Statistical analysis

The lethal concentrations (LC_{50} and LC_{90}) were calculated by regression analysis. Aphid mortalities were corrected using Abbott's formula (Abbott 1925), and corrected mortality proportions were linearized using logit transformation (Dagnelie 1970): $\log it(P) = \ln(P/(1-P))$. A single linear regression was used for modeling the relationship between logit-transformed mortality and logarithm-transformed values of fungal concentrations as explanatory variable: $\log it(P) = \text{slope} \times \ln(\text{concentration}) + \text{intercept}$. The relationship between aphid mortality and spore concentrations was assessed considering Snedecor-F distribution and p values. Reliability of the regression coefficients was assessed using Student's t test.

The lethal times (LT_{50}) were calculated by Kaplan-Meier analysis, and the log-rank test (providing the chi-square and p value) was carried out to check for significant differences between treated and control aphids.

The number of nymphs produced daily per female aphid was expressed as intrinsic growth rate (Wyatt and White 1977) and subjected to the ANOVA-1 test to check for

significant differences between treated and untreated aphids for each time interval.

All analyses were performed with Statistica 9 software. In all the cases, results were considered statistically significant when the p value of the analysis was lower than 5 % ($p < 0.05$).

Results and discussion

Aphid mortality was monitored daily up to 5 days (four replicates). Control mortalities ranged from 3.75 (day 1) to 17.50 % (day 5) for *A. flavus*, from 2.5 to 12.5 % for *A. clavatus* and from 3.75 to 10.00 % for *M. anisopliae*. Corrected percent mortality increased with time interval (day 1 to day 5) and spore concentration (10^3 to 10^7 spores/ml). At day 5, mortality of aphids ranged between 49.90–90.90 % for *A. flavus*, 51.47–84.06 % for *A. clavatus* and 30.37–78.05 % for *M. anisopliae* depending on the spore concentration (Fig. 1). Corresponding lethal concentrations (LC_{50} and LC_{90}) were, respectively, 1.23×10^3 and 1.34×10^7 ($F_{(1,17)} = 59.17$; $p < 0.001$), 4.95×10^2 and 5.65×10^7 ($F_{(1;17)} = 20.44$; $p < 0.001$) and 3.67×10^3 and 9.71×10^7 ($F_{(1,18)} = 26.92$; $p < 0.001$) spores/ml (Table 1). At the highest concentration of 10^7 spores/ml, lethal times (LT_{50}) were reached, respectively, with *A. flavus* at day 2.50 ($\chi^2_{(0.95,1)} = 99.68$; $p < 0.001$), with *A. clavatus* at day 2.85 ($\chi^2_{(0.95,1)} = 92.70$; $p < 0.001$) and with *M. anisopliae* at day 3.26 ($\chi^2_{(0.95,1)} = 80.34$; $p < 0.001$). It was demonstrated that there were rapid declines in aphid populations for 5 days. Regarding the lethal times and concentrations, fungal strains would not have the same pathogenicity. *Aspergillus* isolates induced higher mortalities than *M. anisopliae*, which is a well-known entomopathogen in the literature. These variations might be due to the differences in the virulence of the fungal isolates, the host species or the method used for fungal application. For example, differences in the virulence of four *M. anisopliae* isolates was shown against *Aphis craccivora* (Koch) with LC_{50} ranging from 3.1×10^5 to 7.4×10^6 spores/ml (Ekesi et al. 2000). Similarly, Loureiro and Moino (2006) showed susceptibility differences in *Myzus persicae* (Sulzer) and *Aphis gossypii* (Glover) treated with 10^6 spores/ml of the same *M. anisopliae* isolate with LT_{50} of 2.91 and 3.9 days after treatment. Regarding other studies, our results are similar to those of Saranya et al. (2010) against *A. craccivora*

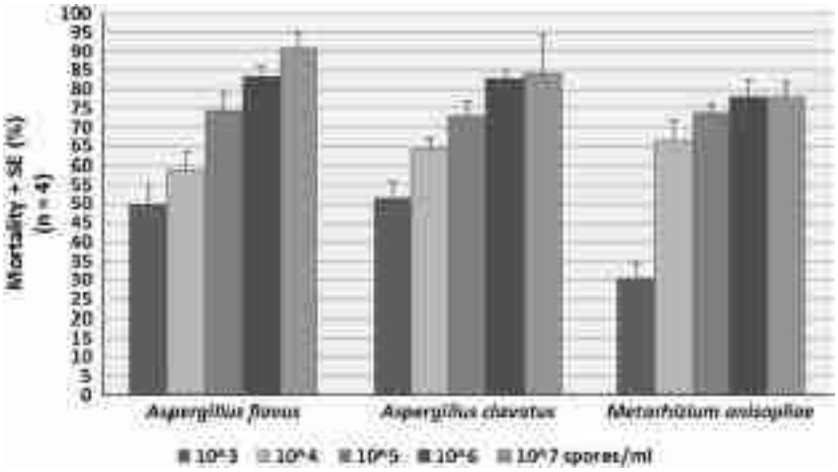

Fig. 1 *Acyrthosiphon pisum* adult female mortality after 5 days of exposure to *Aspergillus flavus*, *Aspergillus clavatus,* and *Metarhizium anisopliae* spores depending on tested concentrations. Values are mean corrected percent mortality of four replicates with standard error (SE)

Table 1 Lethal concentrations (LC_{50} and LC_{90} expressed as spores/ml) of *Aspergillus flavus*, *Aspergillus clavatus,* and *Metarhizium anisopliae* against *Acyrthosiphon pisum* adult females after 5 days of spore exposure

Fungal strain	LC_{50}	LC_{90}	Coefficient	Standard error	*T* test (*df*)	*p* value
Aspergillus flavus	1.23×10^3	1.34×10^7				
Slope			−1.68	0.36	−4.67 (17)	<0.001
Intercept			0.24	0.03	7.69 (17)	<0.001
Aspergillus clavatus	4.95×10^2	5.65×10^7				
Slope			−1.77	0.50	−3.56 (18)	0.002
Intercept			0.22	0.04	5.19 (18)	<0.001
Metarhizium anisopliae	3.67×10^3	9.71×10^7				
Slope			−1.17	0.49	−2.40 (17)	0.028
Intercept			0.19	0.04	4.52 (17)	<0.001

adults for the same treatment time. This was not the case for Yokomi and Gottwald (1988) who reported 100 % mortality of three aphid species [*M. persicae*, *A. gossypii* and *Aphis citricola* (Van Der Goot)] with 10^6–10^7 concentrations of five isolates of *L. lecanii* and *Hirsutella thompsonii* (Fisher) after 4-day treatment.

Postmortem observations revealed mycelia development (corresponding to the inoculated strain) around the dead treated aphids 48 h after incubation in petri dishes for each fungal isolate (Fig. 2). No mycelia appeared from dead control aphids. This suggested that conidia adhered to aphid's cuticle when sprayed and led to their death. Even though *Aspergillus* species such as *A. clavatus* and *A. flavus* are saprophytic fungi, the isolates used in this study might have completed an infectious cycle on aphids as previously reported for other insects. Previously, microscopic observations revealed that *A. clavatus* adhered to and penetrated the cuticle of mosquito larvae (Diptera: Culicidae). Surviving *Culex quinquefasciatus* (Say) larvae that reached the pupal stage produced infected adults (Seye et al. 2009). Similary, surface-inoculated *A. flavus* completed its life cycle on *Bombyx mori* (L.) larvae in 6 to 7 days including hemocoele invasion (Kumar et al. 2004). Moreover, mycelia development and sporulation observed on dead adults could promote fungal persistence in the environment. Aphid reproduction is very fast, and densities could increase rapidly. The autodissemination of spores between adults and their nymphs could be raised as an objective study since the high densities of aphids could increase the contact possibilities among them and consequently horizontal transmission.

Untreated adults produced more nymphs than treated adults. The production of *A. pisum* nymphs decreased with increased conidial concentration for all fungal strains (Fig. 3). The ANOVA-1 test showed that the intrinsic growth rates varied significantly from untreated adults on day 5 ($p = 0.016$) for *A. flavus*, on days 4 ($p = 0.003$) and 5 ($p < 0.001$) for *A. clavatus* and on days 4 ($p = 0.005$) and 5 ($p = 0.011$) for *M. anisopliae*. Many studies showed that entomopathogenic fungi could affect the fecundity of insect species (Castillo et al. 2000; Mulock and Chandler 2001; Quesada-Moraga et al. 2004; Scholte et al. 2006). Our results are consistent with Baverstock et al. (2006), which also revealed that *Pandora neoaphidis* [(Remaudière and Hennebert) Humber] and *B. bassiana* infection appears to have direct effects on adult *A. pisum* reproduction, but no indirect effects on the fitness of their progeny. By contrast, Wang and Knudsen (1993) did not find similar effects with *B. bassiana* against the Russian wheat aphid [*Diuraphis noxia* (Kurdyumov)]. Our results are consistent with the hypothesis that a reduction in aphid fecundity may be related to an increase in pathogen fitness as aphid resources are used for mycelia development and conidia production (Baverstock et al. 2006).

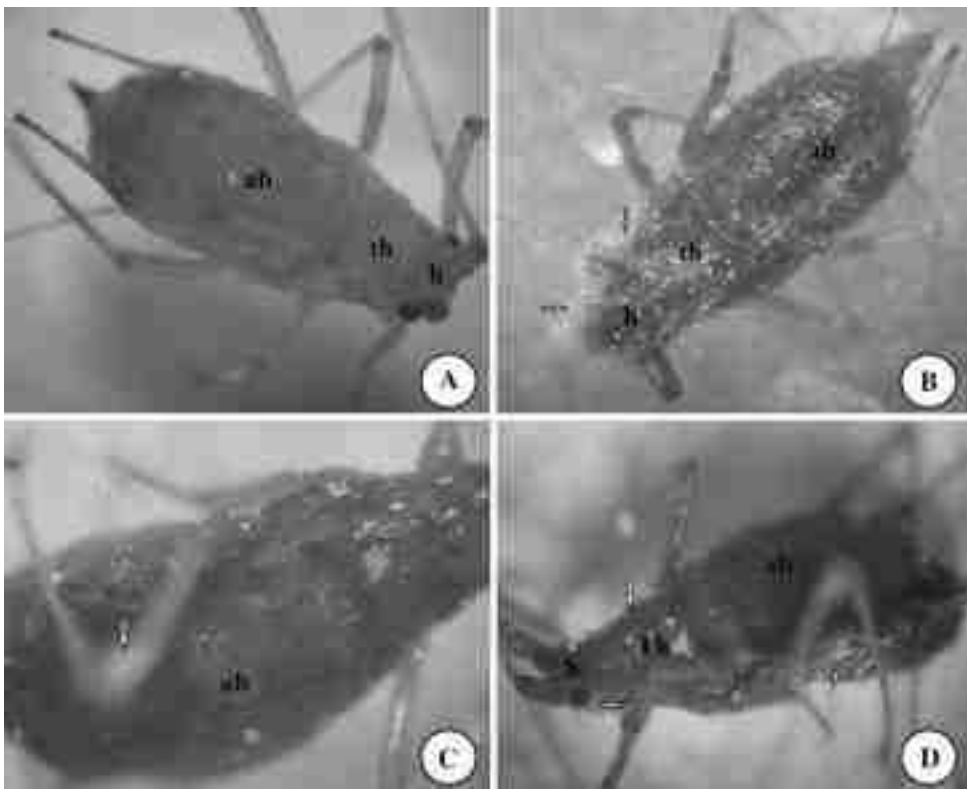

Fig. 2 Non-treated *Acyrthosiphon pisum* (**a**) and treated with *Aspergillus clavatus* (**b**), *Metarhizium anisopliae* (**c**) and *Aspergillus flavus* (**d**) 48 h after incubation on wet paper. Filaments (*arrows*) appeared around the aphid cuticle. Compared to the control (**a**), the treated adults were attacked by fungi. The fungal development was more effective with *A. clavatus* (**b**) than *M. anisopliae* (**c**) and less with *A. flavus* (**d**). *h* head, *th* thorax, *ab* abdomen

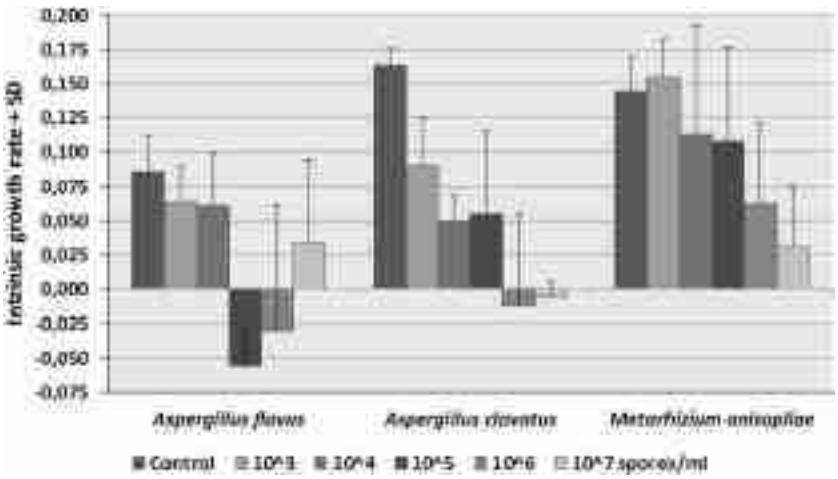

Fig. 3 Intrinsic growth rate [with standard deviation (SD)] in *Acyrthosiphon pisum* adult female populations 5 days after application of different spore concentrations of *Aspergillus flavus*, *Aspergillus clavatus* and *Metarhizium anisopliae*

Aspergillus species display a wide diversity of lifestyles including in clinical, industrial and agricultural environments; some of them may be opportunistic pathogens of a wide range of organisms including agricultural pests (Gibbons and Rokas 2012). To our knowledge, *A. clavatus* and *A. flavus* were reported here for the first time to be pathogenic against aphids. Toxicity tests and postmortem observations suggested that *Aspergillus* species could be useful in aphid control as pest control agents. However, these saprophytic fungi not target only insects, but can also affect immunodepressed humans, mammals and birds (Tell 2005). Non-aflatoxin-producing and non-toxigenic *A. flavus* strains are currently studied in biological control to reduce preharvest contamination of crops with aflatoxin (Ehrlich 2014). Their effects on non-targeted organisms including human health should be investigated.

In conclusion, the adult *A. pisum* aphid was susceptible to the entomopathogenic fungi *A. clavatus, A. flavus* and *M. anisopliae*. Adult mortality increased with time interval and concentration. The reproductive potential of these aphids also decreased with increased concentration. *A. clavatus* and *A. flavus* were here reported for the first time to be pathogenic against aphids. These results suggest that these fungi may be candidates for aphid control. However, toxin extraction, identification and investigation on non-targeted organisms should be performed before use in biological control.

Acknowledgments The authors thank the Islamic Development Bank for the financial support of a Post-Doctoral scholarship on high technologies to Fawrou Seye.

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J. Pestic. Sci. **39**(3), 127–132 (2014)
DOI: 10.1584/jpestics.D14-006

Original Article

Pathogenicity of *Aspergillus clavatus* produced in a fungal biofilm bioreactor toward *Culex quinquefasciatus* (Diptera: Culicidae)

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(Received January 21, 2014; Accepted May 18, 2014)

Many entomopathogenic fungi have been demonstrated to be potential agents for efficiently controlling mosquito populations. In the present study, we investigated a bioreactor system to produce metabolites and conidia by combining technological advantages of submerged and solid-state fermentations. The efficiency of fungal products was tested toward mosquitoes. *Aspergillus clavatus* (Eurotiales: Trichocomaceae) was grown by semi-solid-state fermentation in a bioreactor for up to 7 days. Depending on conidial doses (2.5×10^7, 5×10^7, 7.5×10^7, 10×10^7 and 12.5×10^7 conidia/mL), mortality ranged from 37.2±15.0 to 86.3±5.0% toward larvae and from 35.8±2.0 to 85.2±1.5% toward adults. The metabolites (10, 20, 40, 60, 80 and 100% v/v) yielded mortality from 23.7±15.0 to 100.0±0.1% toward larvae, and two sprayed volumes (5 and 10 mL) reached 45.5±1.4 and 75.6±2.6% mortality, respectively, toward adults.

Keywords: biological control, entomopathogenic fungi, *Aspergillus clavatus*, biofilm bioreactor, solid-state fermentation, submerged fermentation.

Introduction

Mosquitoes (Diptera: Culicidae) are permanent blood sucking vectors of diseases such as dengue, filariasis, and malaria. Many mosquito species and strains are resistant to insecticides commonly used for their control. Biological agents have become increasingly attractive alternative for mosquito control. Among potential microorganisms, entomopathogenic fungi have been used and have provided most interesting results in several pest controls. Conidia are classically applied, but toxic metabolites have also been produced in liquid medium and used against mosquitoes.[1,2] *Metarhizium anisopliae* and *Beauveria bassiana* are promising alternatives to conventional insecticides for eliminating mosquito vectors.[3,4] Recently, many *Aspergillus* species have commonly been used against mosquitoes.[5,6] The larvicidal effect of *Aspergillus flavus* and *Aspergillus parasiticus* metabolites for controlling *Culex quinquefasciatus* has been shown.[7] *Aspergillus clavatus* conidia have also been demonstrated to be efficient against mosquitoes.[8,9] The possibility of using *Aspergillus niger* metabolites against *Cx. quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* larvae has also been shown.[10]

The development of a reliable and efficient fermentation system for large-scale production of insecticidal products is still needed. Classical submerged fermentation of filamentous microorganisms in aqueous suspensions may impair the production of conidia and metabolites of biotechnological interest.[11–13] By contrast, solid-state fermentation is a promising alternative since conidia and metabolites are generally produced in higher quantities when these microorganisms are produced on a solid substrate.[11–16] For example, *Aspergillus oryzae* has been reported to produce a 500-fold higher yield of recombinant chymosin in solid-state fermentation than in submerged fermentation.[17] In this context, efficient utilization of agro-industrial residues as carbon sources has been shown for the mass production of entomopathogenic fungi.[18–21] However, the absence of free water induces parameter variations (such as pH, temperature, moisture, dissolved oxygen or CO_2) that are difficult to control. A complementarity method between solid- and liquid-state fermentations has been attempted in sequential culture.[22]

A fungal biofilm reactor combining the technological advantages of submerged fermentation (*i.e.*, free water facilitating the control of culture parameters) with the biological characteris-

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Published online August 14, 2014

tics found in solid-state fermentation has been established in the present study. Insecticidal activity of conidia and metabolites secreted by an entomopathogenic *A. clavatus* strain in this culture system was shown against *Cx. quinquefasciatus* larvae and adults.

Materials and Methods

1. Fungal strain

An *A. clavatus* (Eurotiales: Trichocomaceae) strain isolated from the locust cricket *Oedaleus senegalensis* (Orthoptera: Acrididae) at the Laboratory of Reproductive Biology of the University Cheikh Anta Diop (Dakar, Senegal) and shown to be pathogenic against *Anopheles gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus* larvae[9] was used (accession number MUCL 55275, Belgian Coordinated Collections of Microorganisms, Mycothèque de l'Université Catholique de Louvain (Belgium)). This strain was cultivated on potato dextrose agar (PDA) in Petri dishes and stored at 4°C.

2. Inoculum

As a preculture, *A. clavatus* mycelia were produced in Erlenmeyer conical flasks (500 mL) containing 300 mL of distilled water, 1% peptone, 1% yeast extract and 2% glucose sterilized at 120°C for 20 min. These cultures were incubated after inoculation for 48 hr on a rotary shaker at 140 rpm and 30°C to produce fungal pellets.

3. Metal structured packing for fungal sporulation

To facilitate pellets fixation and fungal sporulation in a bioreactor, a metal packing was molded. Packing is a cylinder composed of several stainless steel corrugated sheets independent of each other, as previously described.[23] For packing, 28 metal rectangular plates (16×16 to 2×16 cm) with a 2-mm mesh were cut for assembly. The plates were superimposed symmetrically in pairs following the decreasing width of the rectangle in order to form a cylinder approximately 16 cm in diameter. All were then surrounded by a broad plate to form a block of cylindrical packages 16 cm in height and 16 cm in diameter. One of the bases was covered by a circular metal to prevent solid substrates from falling into the liquid medium.

4. Bioreactor system

The fermentation run was carried out in a 20-L bioreactor with 6-L working volume. Wheat bran (200 g) was introduced as a carbon source in the cylinder between the metal plates of the packing, which was suspended in the Biolafitte bioreactor (Fig. 1). Peptone and yeast extract (60 g each) were previously introduced in the total working volume of the reactor containing 6 L of distilled water and 0.05 g/L chloramphenicol as a bacteriostatic agent. The preparation was autoclaved at 120°C. The preculture was aseptically injected into the bioreactor using a syringe. With a peristaltic pump (120 rpm), the culture medium was continuously stirred (connections made with silicone tubing with an internal diameter of 5 mm) and injected on the packing

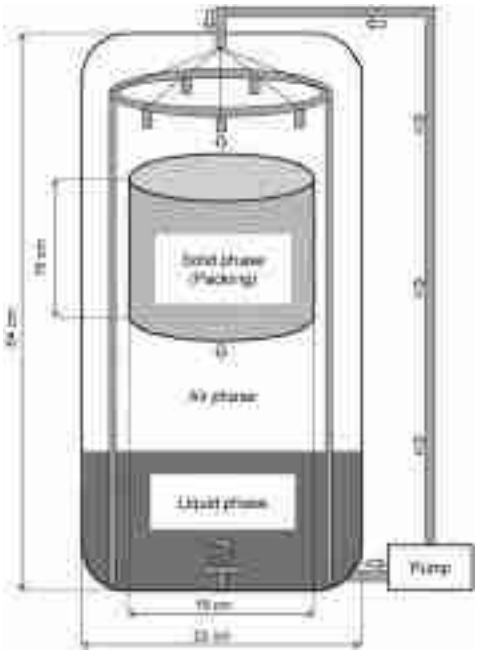

Fig. 1. Schematic diagram of a 20-L bioreactor system with metal packing. The packed structure (16 cm height×16 cm diameter) consisted of a stainless steel cylinder composed of several independent rectangular plates surrounded by a broad plate. The cylinder was closed at the bottom by a circular plate. Uniform liquid media were ensured by mixing. The pump drew the liquid containing the fungal pellets provided from a preculture, and injected them into the packing containing wheat bran as a solid substrate. Biomass immobilization was ensured to avoid liquid viscosity.

containing wheat bran. The mycelial pellets were then carried out with the liquid medium and fixed to the molded system. The temperature was maintained at 30°C by circulating temperature-controlled water. Air was continuously supplied to the bioreactor at 3 L/min. The pH was allowed to vary freely and was recorded continuously. Cultivation was carried out for 7 days.

5. Conidia recovery

After incubation, the packing that contained fungal conidia and biomass was removed, and the liquid medium was stored at −80°C. The packing was washed with an aqueous solution containing 0.05% Tween 80 (0.05% Tween 80 solution) to detach the conidia. The washing solution was then filtered to discard impurities and centrifuged (4°C, 3,000 *g*, 5 min). The conidia were finally suspended in a 0.05% Tween 80 solution and the dose was adjusted to 10^9 conidia per mL using a hemocytometer (Thoma®).

6. Filtration of metabolites

Liquid medium from the bioreactor was centrifuged (4°C, 7,500 *g*, 30 min) to eliminate impurities. Medium was then filtered with ultrafiltration membranes (Prep/Scale-TFF-1 Cartridge (Millipore) 5.8 cm in diameter and 15.2 cm long with polyethersulfone membrane filtration (column 1 ft2 cartridge))

to remove unconsumed nutrients from the culture medium. Final concentrated metabolites (800 mL) with high molecular weight products (MW>100,000) were obtained and stored at −80°C.

7. Mosquito rearing

Cx. quinquefasciatus (S-Lab) adults were reared in 50-×50-×50-cm cages and fed with a 10% sucrose solution. Blood meal was made using the Hemotek feeding system. Larvae were maintained in distilled water (25-×15-×5-cm containers) in laboratory conditions (25±2°C, 70±5% relative humidity, and 16:8 hr (Light:Dark) photoperiod) and fed with fish food (TetraMin®) and natural brewer's yeast tablets (Biover®).

8. Bioassays

8.1 Larval treatment

First, groups of 20 third-instar larvae were exposed to conidial doses of 2.5×10^7, 5×10^7, 7.5×10^7, 10×10^7 and 12.5×10^7 conidia/mL in separate bottles for 3 days. Control larvae were maintained in 0.05% Tween 80 solution. Second, larvae were exposed to serial dilutions (10, 20, 40, 60, 80, and 100% (v/v)) of concentrated metabolites with distilled water for 3 days. Control larvae were maintained in distilled water. All larvae were fed with the same food as used for rearing.

8.2 Adult treatment

Conidia were formulated with 5% sunflower oil (v/v) in 0.05% Tween 80 solution. The same conidial suspension series used against larvae were directly sprayed on a total of 20 three- and five-day-old sugar-fed adults that were transferred in 25-×25-×25-cm mosquito netting cages and kept for 3 days. For control, a 0.05% Tween 80 solution containing 5% sunflower oil (v/v) was sprayed on the adults. In parallel, 5 and 10 mL of concentrated metabolites were sprayed directly on the groups of 20 adults previously introduced into separate cages and kept for 3 days. For control, distilled water was sprayed on the adults.

8.3 Statistical analysis

Four replicates were performed for each treatment for different durations with new independent fermentation products. Mortality was recorded daily for three days and corrected using Abbott's formula.[24] Dead larvae were removed, rinsed with sterile distilled water to eliminate non-attached conidia, and examined under a microscope for fungal infection. Dead larvae and adults were incubated in Petri dishes containing wet filter paper to monitor the conidial germination. All experiments were done in laboratory conditions with 25±2°C temperature and a 16-hr light photoperiod.

Results

1. Pathogenicity of conidia on Culex quinquefasciatus

Conidia were effective against *Cx. quinquefasciatus* larvae and adults. The percentage of larval mortality increased with increasing conidial concentration and ranged from 37.2±15.0 to 86.3±5.0% for 72 hr of treatment (Table 1). Adult mortality ranged from 35.8±2.0 to 85.3±1.5% after 72 hr post-inoculation (Table 2). Conidial adhesion to the larval cuticle was not observed. Nevertheless, conidia ingested by larvae during bioassays invaded the larval gut, germinated, and emerged thereafter from dead larvae (Fig. 2A–C). By contrast, the conidia penetrated the adult mosquito's cuticle before emerging (Fig. 2D).

2. Toxicity of metabolites on Culex quinquefasciatus

Metabolites produced by *A. clavatus* in a bioreactor showed tox-

Table 1. Mortality of *Culex quinquefasciatus* larvae treated with *Aspergillus clavatus* conidia produced in bioreactor*

Treatment time	Conidial concentration (10^7 conidia/mL)				
	2.5	5	7.5	10	12.5
Cumulative mortality (%)±SD					
24 hr	15.8±14.0	17.1±17.0	30.3±20.0	38.2±15.0	60.5±15.0
48 hr	25.0±6.0	35.5±22.0	42.1±30.0	47.4±9.0	77.6±13.0
72 hr	37.2±15.0	55.3±5.0	65.8±9.0	73.8±6.0	86.3±5.0

* Values are average corrected mortality (%)±standard deviation (SD).

Table 2. Mortality of *Culex quinquefasciatus* adults treated with *Aspergillus clavatus* conidia produced in bioreactor*

Time after inoculation	Conidial concentration (10^7 conidia/mL)				
	2.5	5	7.5	10	12.5
Cumulative mortality (%)±SD					
24 hr	20.0±3.0	20.0±4.0	24.8±2.0	28.3±8.0	37.5±1.0
48 hr	25.8±5.0	55.7±2.0	50.0±0.2	60.0±3.1	75.7±0.8
72 hr	35.8±2.0	56.5±0.9	65.5±0.7	75.0±14.0	85.3±1.5

* Values are average corrected mortality (%)±standard deviation (SD).

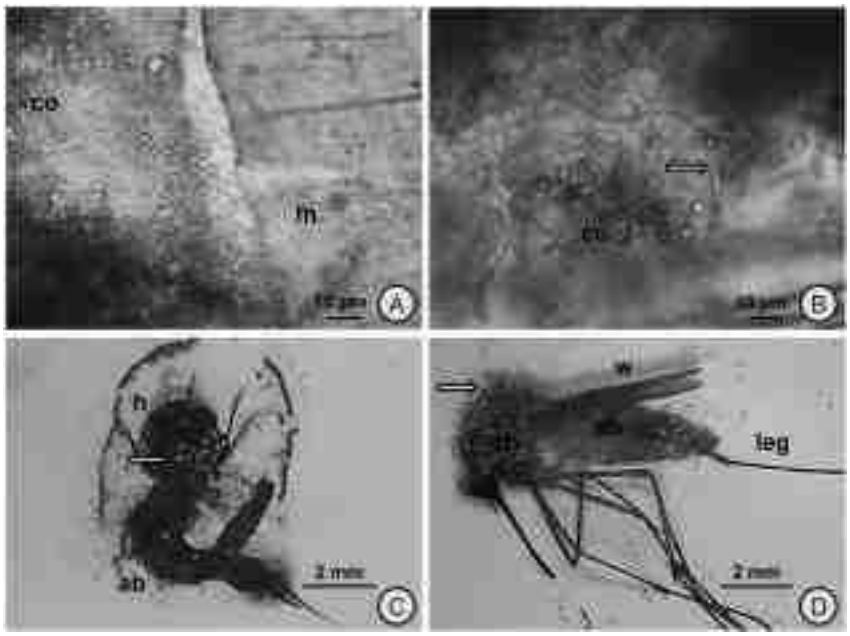

Fig. 2. *Culex quinquefasciatus* larvae and adults infected with *Aspergillus clavatus* cultivated in the bioreactor system. Conidia (A) were observed within the larval gut after 24 hr of treatment. Germ tube (arrow) and filaments within the larval gut (B), and conidial heads (arrow) on the larval cadavers (C) were observed 72 hr after treatment. Conidial formation was observed on the thorax (arrow), legs, and wings of the adults 72 hr post-inoculation (D). co: conidia, m: muscle, h: head, ab: abdomen, th: thorax and: wing.

Table 3. Effect of *Aspergillus clavatus* metabolites on *Culex quinquefasciatus* larvae*

Treatment time	Concentration of metabolites (%)					
	10	20	40	60	80	100
Cumulative mortality (%)±SD						
24 hr	2.6±3.0	22.4±16.0	27.6±10.0	67.1±8.0	76.3±6.0	93.4±5.0
48 hr	11.8±13.0	26.3±15.0	38.2±15.0	73.7±4.0	78.9±4.0	97.4±3.0
72 hr	23.7±15.0	39.5±27.0	55.3±5.0	76.3±6.0	82.9±2.0	100.0±0.1

* Values are average corrected mortality (%)±standard deviation (SD).

Table 4. Effect of *Aspergillus clavatus* metabolites on *Culex quinquefasciatus* adults*

Time after treatment	Sprayed doses of metabolites (mL)	
	5	10
Cumulative mortality (%)±SD		
24 hr	7.7±0.1	18.5±0.2
48 hr	40.1±2.0	65.3±3.6
72 hr	45.5±1.4	75.6±2.6

* Values are average corrected mortality (%)±standard deviation (SD).

icity against *Cx. quinquefasciatus* larvae and adults. The mortality increased with increasing metabolite concentration and ranged from 23.7±15.0 to 100.0±0.1% for larvae after 72 hr post-treatment (Table 3) and 45.5±1.4 to 75.6±2.6% for adults after 72 hr of treatment (Table 4).

Discussion

The system developed in this work shows the possibility to produce simultaneously, with controlled parameters, two kinds of *A. clavatus* products: conidia and metabolites in a bioreactor. The substrate contained in the metal packing allowed for efficient growth of the fungal biomass, which sporulated. The liquid media allowed for easy recovery of metabolites secreted by the fungal biomass. Moreover, the biomass remained confined on the substrate and did not increase the viscosity of the liquid medium. The present process highlights the possibility to control the parameters of metabolites and conidia production for further study. Homogenization of the physical traits in the packed structure (pH, oxygen, temperature), as well as their impact on conidia and metabolites production, must be investigated. Other entomopathogenic fungi, such as *M. anisopliae* and *B. bassiana*, could also be cultivated in this bioreactor system for industrial

production.

Conidia and metabolites were both found to be effective for controlling *Cx. quinquefasciatus* larvae and adults. Unexpectedly, conidia cultivated on wheat bran were not strongly pathogenic toward *Cx. quinquefasciatus* larvae as compared with approximately the same dose of conidia cultivated on wheat powder.[9] Less virulence may be related to the kind of substrate used for fungal production as previously discussed.[25] However, the pathogenicity of the conidia toward adult mosquitoes was not decreased as compared with previous results, where 86.0± 1.7% mortality at day 5 was obtained with 7.9×10^7 conidia/mL.[8] The sunflower oil used for the fungal formulation facilitated the conidial adhesion on the adult cuticle as Neem oil against *Cx. quinquefasciatus*.[8] The observation of dead adults and larvae confirmed that the mosquito mortality was due to the conidia that, respectively, adhered to and penetrated the cuticle or invaded the larval gut. In the latter case, larval death may be due to toxins secreted during germination in the gut of larvae, as the digestive tract was previously reported to be a significant infection site.[26,27]

Our results are similar to those of other studies that showed the toxicity of fungal metabolites against mosquitoes.[2,28,29] *A. clavatus* metabolites were found to be toxic toward adults and larvae with significant mortality rates after 72 hr. Regarding the application approach, metabolites could act by contact with adults and also by ingestion by larvae. Another study revealed that metabolites of fungi, such as *Tolypocladium inflatum* (Hypocreales: Ophiocordycipitaceae), could attack mosquito larvae and be the main cause of histopathological damages.[30] *A. clavatus* germination was associated with the secretion of some toxins.[31–33] These suggest that metabolites produced by the fungus act on mosquito tissues including the midgut of larvae.[9,30,34] These metabolites must be purified and their chemical structure identified. Moreover, the mechanism of the action of these metabolites must be defined on non-target organisms before their use for general biocontrol in the field.

Conclusion

The present study shows the high potential utility of fungal production in a liquid phase continuously recirculated on a metal structured packing in a bioreactor. This new system was developed for fungal growth and metabolite production, combining the technological advantages of submerged and solid-state fermentations. The process allowed facility in recuperation and purification of conidia (confined on the solid substrate) and metabolites (contained in the liquid medium). *A. clavatus* conidia and metabolites were virulent and could be a promising alternative to chemical control of mosquito larvae and adults. Therefore, it is necessary to isolate and show the effect on non-target organisms before use. These results also highlight the possibility to control the culture parameters for further studies. The homogenization of these physical traits in the packed structure, as well as their impact on conidia and metabolites production, must now be investigated.

Acknowledgements

The authors thank the Islamic Development Bank who supported this work *via* a post-doctoral fellowship to Fawrou Seye for High Technology in Gembloux Agro-Bio Tech (University of Liege).

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Academia Journal of Biotechnology 1(3): 046-052, March 2013
http://www.academiapublishing.org/ajb
ISSN: 2315-7747

Research Paper

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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Accepted 13 March, 2013

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

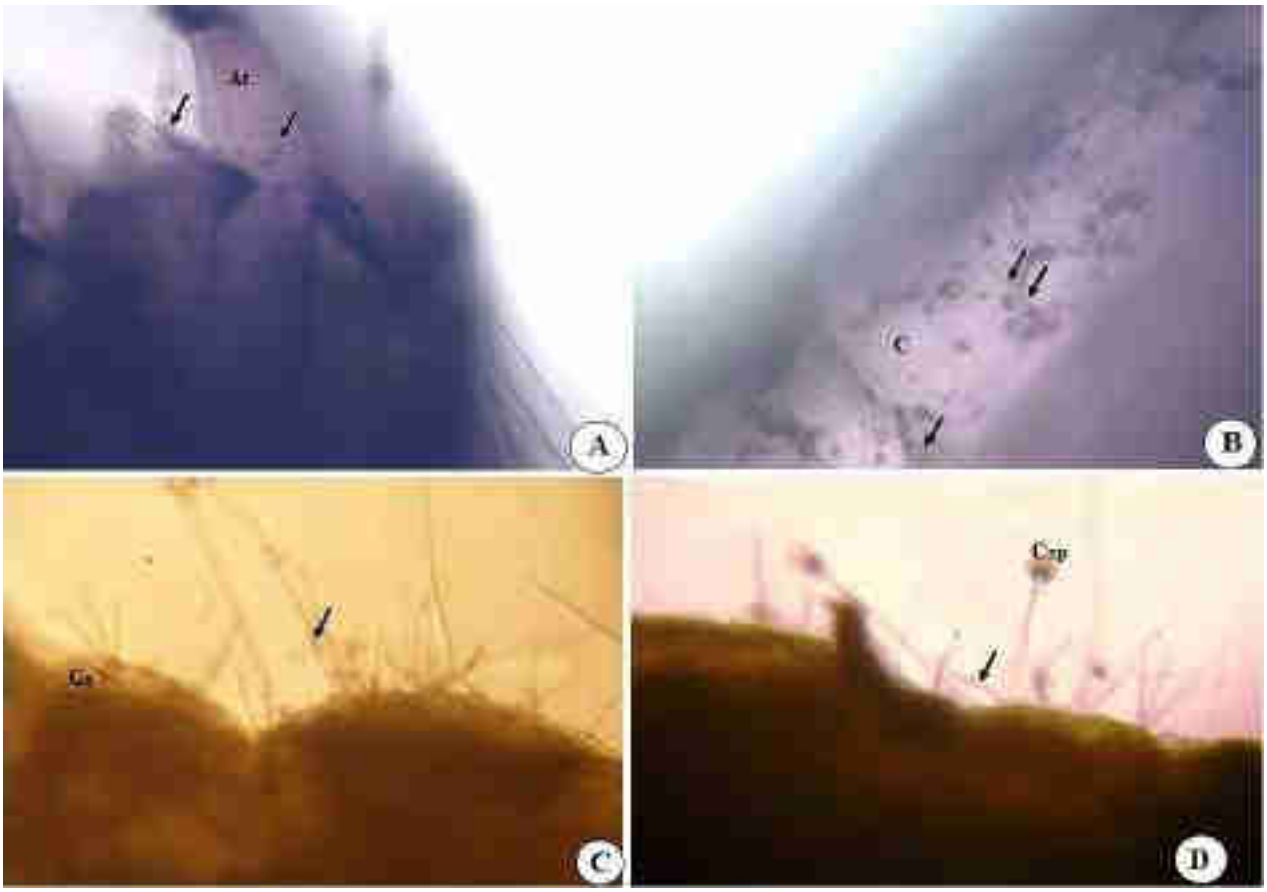

Figure 1. *Anopheles gambiae* larvae infected by *Metarhizium anisopliae* formulated with neem oil (Suneem) 24 h (A and B), 48 h (C**)** and 72 h (D) after treatment (x 400). C= cuticle, At. = antenna, Csp= conidial spore, Cs= comb. Conidia were adhered (arrows) on the head (A) and abdomen (B) in 24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

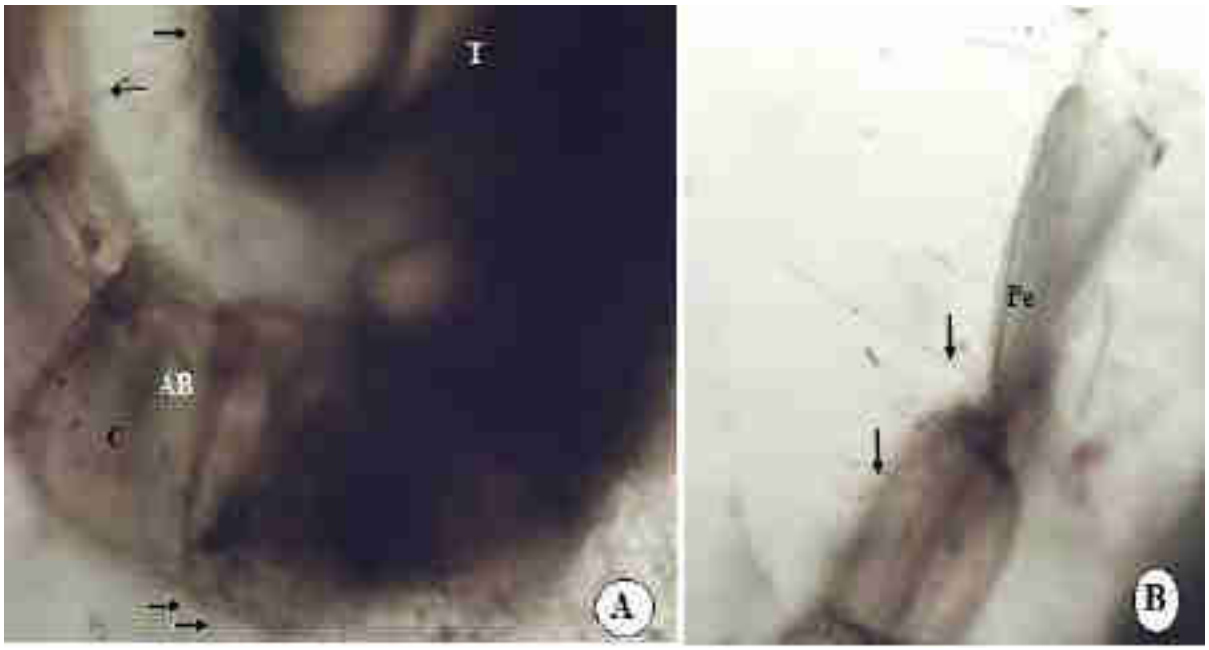

Figure 2. *Anopheles gambiae* pupae infected by *Metarhizium anisopliae* formulated with neem oil (Suneem) 48h after treatment (A and B) (x400). AB= abdomen, C= cuticle, Pe= paddles, T= thorax. Mycelia germination was observed on the pupae (arrows) at the thorax and abdomen.

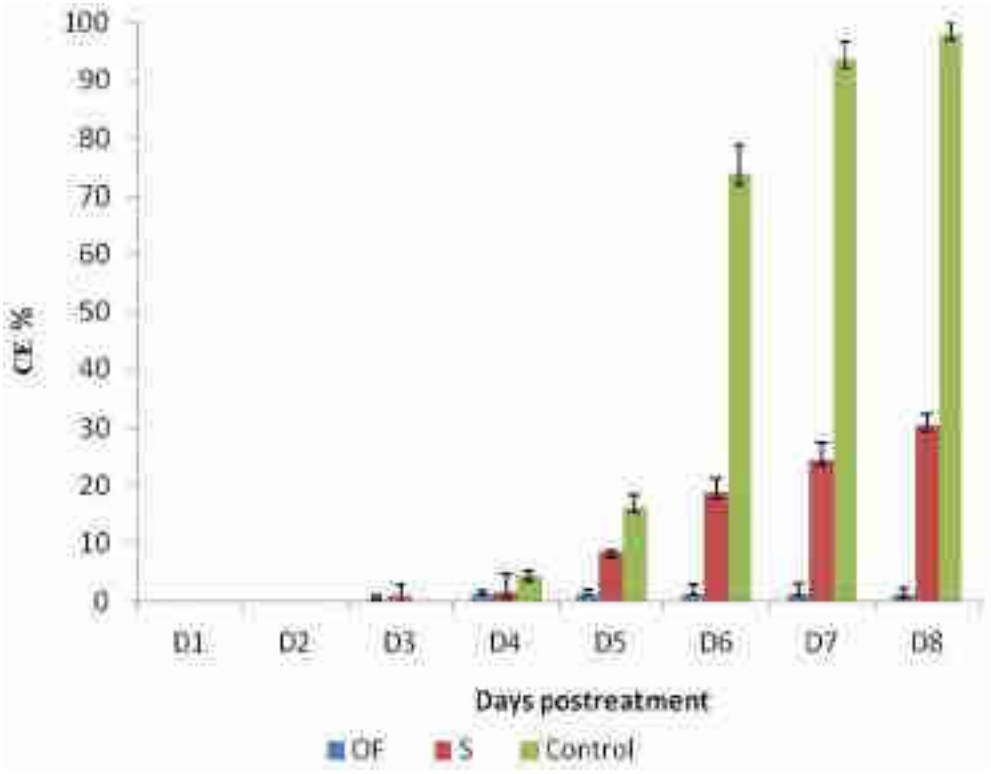

Figure 3. Percent of cumulative emergence of *Anopheles gambiae* s.l. adults treated with *Metarhizium anisopliae* oil formulation in rainy season. The environmental temperature mean was 28 °C and 25 °C for the water vats. Percent of emergence inhibition are more significant with oil formulation than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

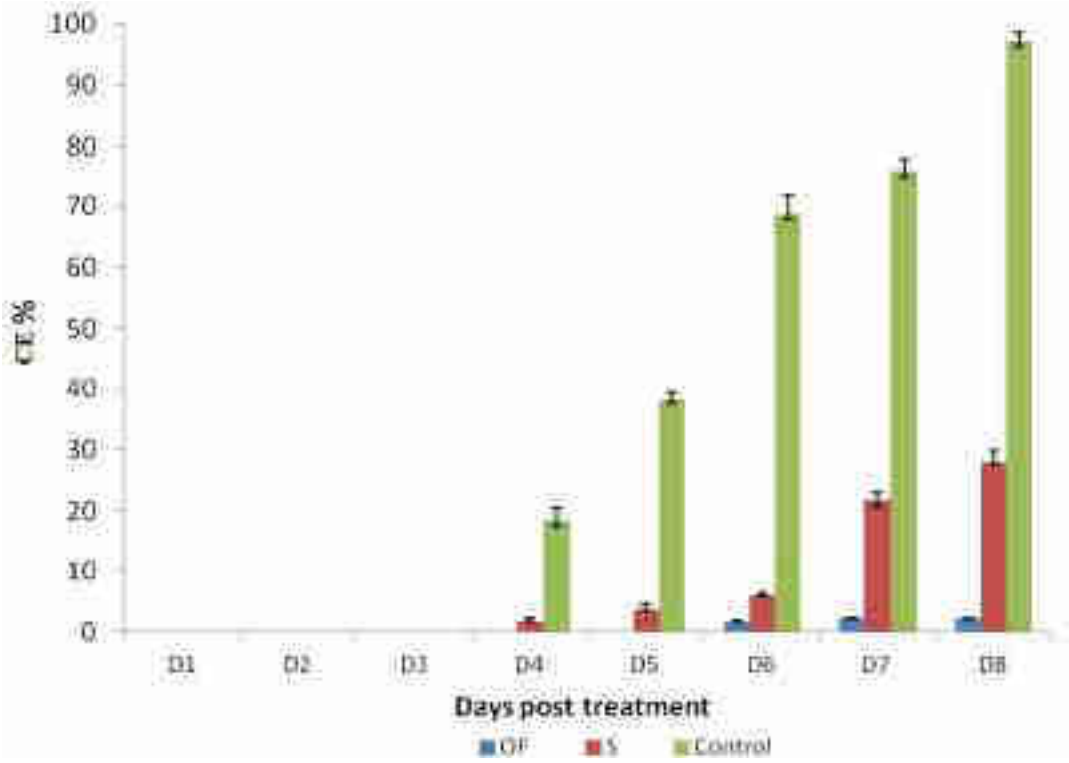

Figure 4. Percent of cumulative emergence of *Anopheles gambiae* s.l. adults treated with *Metarhizium anisopliae* oil formulation in dry season. The environmental temperature mean was 27°C and 24°C for the water vats. T-test reveal that Percent of emergence inhibition are more significant with oil formulation 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.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Scientific Research of Senegal in the project program “FIRST” 2008. We thank also the Islamic Development Bank (BID) for the training fellowship in Belgium.

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Cite this article as:

Seye Fawrou, Ndione Raymond Demba, Touré Mamour, Ndiaye Mady, Boukraa Slimane, Bawin Thomas, Zimmer Jean-Yves and Francis Frédéric (2013). Effect of humic and application at different growth stages of kinnow mandarin (citrus reticulata blanco) on the basis of physio-biochemical and reproductive responses. Acad. J. Biotechnol. 1(1): 046-052.

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

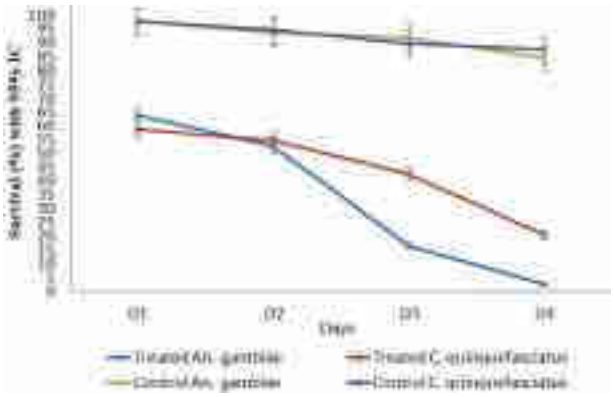

Figure 1: Effect of *Metarhizium anisopliae* in Suneem (neem oil) formulation on *Anopheles gambiae* and *Culex quinquefasciatus* survival. For the control, the mosquitoes are treated with Suneem.

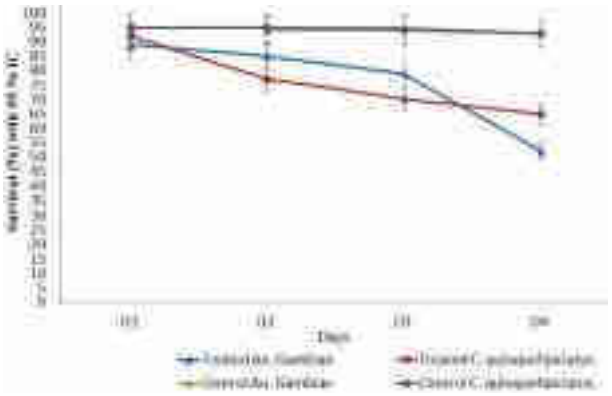

Figure 2: Effect of *Metarhizium anisopliae* in water formulation on *Anopheles gambiae* s.l. and *Culex quinquefasciatus* survival. For the control, the mosquitoes are not treated with fungus.

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

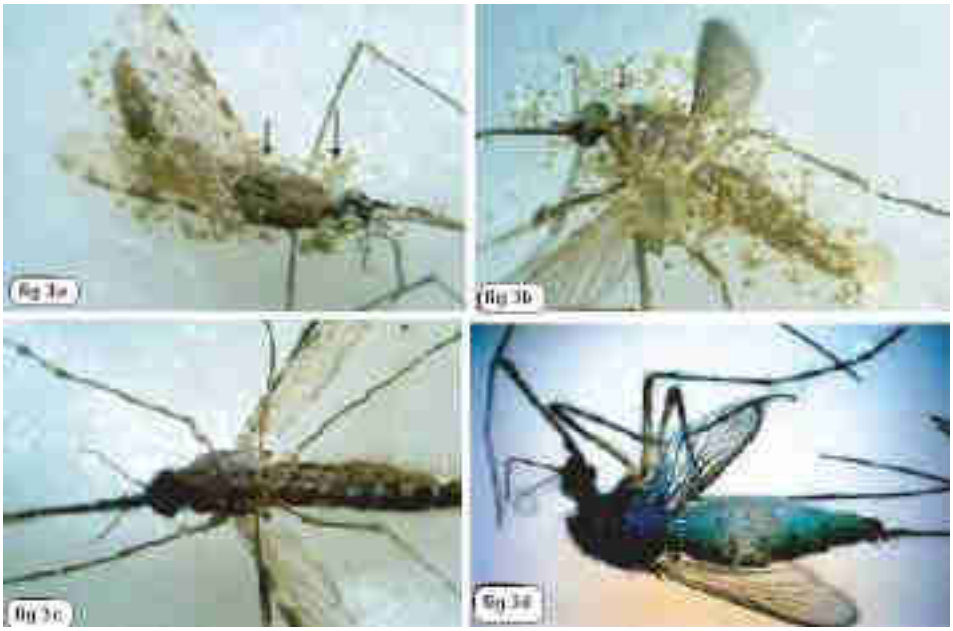

Figure 3: *Anopheles gambiae* (a) and *Culex quinquefasciatus* (b) mosquito adults infected by *Metarhizium anisopliae* and none infected (control) (c,d).

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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Pathogenicity of the fungus, *Aspergillus clavatus*, isolated from the locust, *Oedaleus senegalensis*, against larvae of the mosquitoes *Aedes aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus*

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Abstract

The use of insect pathogenic fungi is a promising alternative to chemical control against mosquitoes. Among the Hyphomycetes isolated from insects for mosquito control, the genus *Aspergillus* remains the least studied. In September 2005, four fungi were isolated from the Senegalese locust, *Oedaleus senegalensis* Kraus (Orthoptera: Acrididae), collected in Dakar, Senegal. One of these fungi, identified as *Aspergillus clavatus*, Desmazières (Eurotiales: Trichocomaceae) was highly pathogenic against larvae of the mosquitoes *Aedes aegypti* L., *Anopheles gambiae* s.l. Giles and *Culex quinquefasciatus* Say (Diptera: Culicidae). An application of 1.2 mg/ml dry conidia yielded 100% mortality after 24 hours against both *Ae. aegypti* and *Cx. quinquefasciatus* while with *An. gambiae* it was 95%. With unidentified species in the genus *Aspergillus*, mortality after 24 h was <5% against all the larval species. Application of *A. clavatus* produced in a wheat powder medium using doses ranging between 4.3 to $21x10^7$ spores/ml, caused 11 to 68% mortality against *Cx. quinquefasciatus* at 24h, and 37 to 100% against *Ae. aegypti*. Microscopic observations showed fungal germination on both *Ae. aegypti* and *Cx. quinquefasciatus* larvae. Histological studies revealed that *A. clavatus* penetrated the cuticle, invaded the gut and disintegrated its cells. Some *Cx. quinquefasciatus* larvae, treated with *A. clavatus* reached the pupal stage and produced infected adults. However, the infection was mainly located on the extremity of their abdomen. These results suggest that *A. clavatus* could be an effective tool to manage mosquito proliferation.

Keywords: biological control, entomopathogenic fungi
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Received: 25 December 2007 | **Accepted:** 3 March 2008 | **Published:** 2009
Associate Editor: Fernando Vega was editor of this paper

ISSN: 1536-2442 | Volume 9, Number 42

Cite this paper as:
Seye F, Faye O, Ndiaye M, Njie E, Afoutou JM. 2009. Pathogenicity of the fungus, *Aspergillus clavatus,* isolated from the locust, *Oedaleus senegalensis,* against larvae of the mosquitoes *Aedes aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus*. 7pp. *Journal of Insect Science* 9:42, available online: insectscience.org/9.42

Introduction

Mosquito-borne diseases currently represent a great health threat in tropical and subtropical climates. As an alternative to chemical insecticides, natural products (Koua, 1998; Batra et al., 1998; Ravindra et al., 2002), predatory fish (Legner 1995; Karch and Coz 1983; Fillinger et al. 2003) and entomopathogenic fungi (Nnakumusana 1985; Su et al. 2001; Scholte et al. 2003) are frequently used in mosquito control. Fungi such as *Metarhizium anisopliae* (Metchnikoff) Sorokin (Moniliales) and *Beauveria bassiana* (Balsamo) Vuillemin (Clavicipitaceae), commonly found on terrestrial insects can also kill mosquito larvae (Alves et al. 2002; Silva et al. 2004a). Studies have shown that mosquito larvae are susceptible to infections by fungi such as *Leptolegna chapmanii* (Lord and Fukuda, 1988), *M. anisopliae* (Riba et al. 1986; Lacey et al. 1988; Alves et al. 2002; Silva et al. 2004a; Wright et al. 2005), *B. bassiana* (Alves et al. 2002), *Aspergillus parasiticus* (Hati and Ghosh 1965), *Aspergillus* spp. (de Moraes et al. 2001), *Aspergillus flavus*, *A. parasiticus*, *Penicillium falicum*, *Fusarium vasinfectum* and *Trichoderma viride* (Govindarajan et al. 2005). Among the various *Aspergillus* species known to infect mosquitoes, *A. clavatus* Desmazières (Eurotiales: Trichocomaceae) has not been examined as a possible biological control agent. This study assessed the pathogenicity of an *A. clavatus* strain against larval stages of various species of mosquitoes.

Materials and Methods

Isolation

In September 2005, locusts of the species *Oedaleus senegalensis* Kraus (Orthoptera: Acrididae) were collected from plants growing near the department of Animal Biology (University CHEIKH A. DIOP, Dakar- Senegal). They were killed and placed for 24 hours on soil collected from the botanical garden to allow saprophytic fungi attack. Afterward, they were placed in Petri dishes containing 20 mg of wheat flour (locally purchased and sterilized in the autoclave for about 15 min at 120°C) mixed with 15 ml of sterile distilled water. One Petri dish containing the same medium only was used as a control. They were maintained at 26°C average temperature and in the range of 80– 85% ambient RH. Four days later, 4 fungal isolates appeared in the plates containing the insects, but not on the control. These fungi were separately cultivated in Petri dishes on the same medium. Dry conidia were harvested from the surface of the medium directly by scraping and conserved in Pyrex bottles sterilized at 120°C.

Fungi were identified according to Rapper and Fennel (1965), Samson (1979) and Guarro et al. (1999). The fungus *A. clavatus* was easily identified by his long phototrophic conidiophores (Yaguchi et al., 1993) on microscopic examination during germination.

Preliminary fungal tests on the mosquito larvae (Bioassay 1)

In September 2005, larvae of *Aedes aegypti* L., *Anopheles gambiae* s.l. Giles and *Culex quinquefasciatus* Say (Diptera: Culicidae) were collected from various vats containing rainwater. For each fungal isolate, 1.2 mg/ml dry conidia were applied to 25 larvae (3^{rd} and 4^{th} instar) in 9 X 1.5 cm Petri dishes sterilized at 120°C and containing sterile 25 ml of tap water. There were four replicates for each treatment. Four non-treated Petri dishes served as control. The more virulent *A. clavatus* fungus was selected for production and application.

Microscopic observations

Larvae of *Ae. aegypti* and *An. gambiae* treated with *A. clavatus* were fixed after dying, sectioned, mounted, and then observed under light microscopy. The larvae of *Cx. quinquefasciatus* treated with *A. clavatus* at 1.2 mg/ml were used for cuticle observations under light microscopy. Larvae treated with three drops of the aqueous spore solution (1.2 mg/ml), were incubated on wheat powder medium (for *Ae. aegypti*) and on wet filter paper (for *Cx. quinquefasciatus*) at 85% R.H. and 26°C followed by observation of fungal germination.

Fungal production

A. clavatus was grown in Petri dishes containing 20 g of wheat powder (sterilized for 15 min at 120°C) and 15 ml of sterile distilled water. After four days incubation at 26°C, the substrate and dry conidia content were mixed to obtain a powder (conidia - wheat powder mixture). The number of conidia was determined using a haemocytometer.

Application of fungus (Bioassay 2)

Ae. aegypti and *Cx. quinquefasciatus* mosquito larvae were collected from vats containing rainwater in late November 2005. *An. gambiae* larvae were scarce at this time and were not used for the bioassay. Six plastic bottles (10 x 10 x 7 cm), each containing 500 ml of sterile tap water and 50 larvae (L3 and L4), were used for each mosquito species. Larvae were then treated with *A. clavatus* conidial mixture at 4, 8, 12, 16 and 20 g/l. The corresponding concentrations were 4.3; 8.5; 13; 17 and 21 x 10^7 spores/ml respectively. Culture medium (wheat powder only) at 0.02 mg/ml served as control for each treatment. Total mortality was recorded for all replicates of each treatment at 24 hours post-inoculation. The surviving larvae were reared in plastic boxes (10 x 10 x 7 cm) containing 500 ml of tap water for 7 days. The emerging adults were incubated for 24 h at 26°C and fungal germination was observed after microscopic examination.

Data analysis

Data on mortality were corrected with Abbott's formula (Abbott, 1925). Student's t-test was used to compare mortality for *Ae. aegypti* and *Cx. quinquefasciatus*.

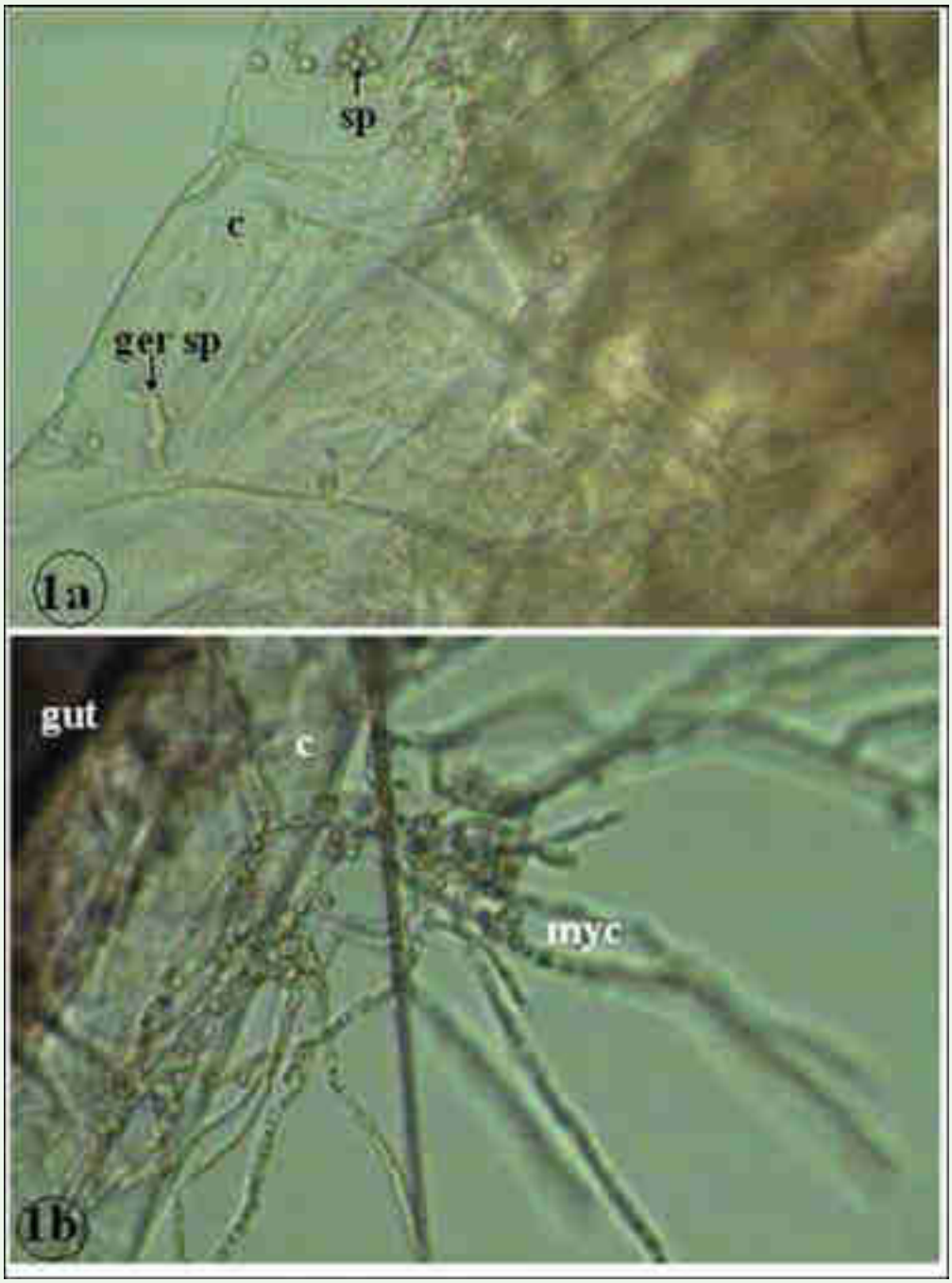

Figure 1. Larvae of *Culex quinquefasciatus* infected by *Aspergillus clavatus* via cuticle (1a and 1b). x 400. c = cuticle, ger sp = germinating spore, myc = mycelium, sp = spore.

Results

Four *Aspergillus* species were isolated from the dead locusts. Preliminary tests (Bioassay1) with conidia suspensions of each isolate revealed that *A. clavatus* was highly pathogenic against larvae of *Ae. aegypti*, *Cx. quinquefasciatus* and *An. gambiae*. Mortality rates were 100% against both *Ae. aegypti* and *Cx. quinquefasciatus*, while against *An. gambiae* it was 95%. All rates were in comparison to the control mortality (< 5%) after 24 hours (Table 1). With the other isolates (S1, S2 and S4), also identified as species in the genus *Aspergillus*, infection against the larvae was less than 5%.

A. clavatus induced significant mortality against mosquito larvae (Table 2) when applied (Bioassay 2 in relative humidity ranging from 65 to 80%, and temperature ranging from 24 to 26°C,. The mortalityt varied from 10.6 to 68% for *Cx. quinquefasciatus* and 36.7 to 100% for *Ae. aegypti*. Larval mortality was significantly higher against

Ae. aegypti than *Cx. quinquefasciatus* (P = 0.0001) (Table 2). *A. clavatus* infection was observed under the microscope. On dead larvae, spores were found attached to the cuticle of *Cx. quinquefasciatus* (Figure 1a). Germinating spores (Figure 1a) and mycelia (Figure 1b) were found growing on *C. quinquefasciatus* larva.

Table 1. Larval mortality 24 h after fungal test on the mosquito larvae Aedes aegypti, Anopheles gambiae and Culex quinquefasciatus (average of four replicates).

	Fungal stocks				
Species	**S1**	**S2**	**S3**	**S4**	**Control**
Aedes aegypti	0	0	100	0	0
Anopheles gambiae	0	0	95	0	0
Culex quinquefasciatus	0	4	100	0	0

Histological studies revealed that the gastric caeca of some *An. gambiae* was invaded by *A. clavatus* spores (Figure 2a). Gut invasion by conidia and initial stages of germinating conidia were observed on *Ae. aegypti* larvae (Figure 2d). The fungus penetrated the cuticle of *Ae. aegypti* larvae. Conidial germination was also observed on *Cx. quinquefasciatus* larvae incubated on wet filter paper and larvae of *Ae. aegypti* incubated on sterile wheat flour. Fungal growth was observed on all treated and incubated larvae.

Occasionally, a low percentage of *Cx. quinquefasciatus* larvae treated with *A. clavatus* conidia were able to pupate and produce adults. Germinating conidia was observed on the tip of adult abdomen 24h after incubation.

Discussion

A. clavatus was more virulent to the mosquito larvae than the other three fungal isolates. Laboratory results showed that, *A. clavatus* was highly pathogenic against larvae of *Ae. aegypti, An. gambiae* and *Cx. quinquefasciatus*. However, these mosquito larvae do not have the same susceptibility to the fungus. With the same dry conidial dose, (1.2 mg/ml), death rate was 100% against both *Ae. aegypti* and *Cx. quinquefasciatus* larvae and 95% against *A. gambiae* larvae. Referring to larval species susceptibility, the effect of this fungus is similar to that of *M. anisopliae* (Moniliales) and *Tolypocladium cylindrosporum* (Hypocreales) against *An. stephensi, Cx. pipiens* and *Ae. aegypti* larvae (Riba et al., 1986).

When treated with *A. clavatus* spores, mortality was 68 % against *Cx. quinquefasciatus* and 100% against *Ae. aegypti* for 21 $x10^7$ spores/ml. Bisht et al. (1996) found that the fungus *Leptolenia caudata* (Oomycetes) yielded a $LD_{100}$of 7.10^3 spores/ml against *An. culicifacies* after 7 days. Riba et al. (1986) obtained a LD_{100} in the order of 10^7 spores/ml with a stock of *M. anisopliae* against *Ae. aegypti* larvae within 26 hours. From our results the LD_{100} against *Ae. aegypti* was closer to that of *M. anisopliae* against *Ae. aegypti*. Observations of larvae treated with *A. clavatus* revealed that just after adhesion of conidia on the cuticle, some germinated. The conidial proliferation on the cuticle became more obvious after 48 hours that is similar to previous studies on insects (Brett *et al.* 2004). Silva et al. (2004a) showed that larvae of mosquito treated with *M. anisopliae* had high amounts of conidia adhering to the colloid chitin with at least 90 % germination after 24 hrs incubation.

High levels of germination occurred on *A. clavatus* on dead *Ae. aegypti* larvae incubated on medium for 48 to 72 hours. Fungal germination was also observed on *Cx. quinquefasciatus* larvae in contact with aqueous solution of *A. clavatus* spores and incubated on wet filter paper, which is in agreement with Silva et al. (2004b).

However, the cuticle does not represent the only way for fungal infection. Other possible routes of invasion for *M. anisopliae* have been identified in mosquitoes via the respiratory siphon or the alimentary canal (Al-Aidroos and Roberts, 1978; Lacey et al., 1988; Silva et al., 2004b). Our histological results revealed a high gastric caeca

Table 2. Percent mortality rates for Culex quinquefasciatus and Aedes aegypti larvae treated with mixture of Aspergillus clavatus (average of four replicates).

When grown in wheat flour, after conidial production by *A. clavatus* the concentration of the homogenous mixture was 10.7 x 10^9 spores/g.

	Dose (10^7 spores/ml)					
Species	**4.3**	**8.5**	**13**	**17**	**21**	**control**
Culex quinquefasciatus	10.6 ± 0.5	46.3± 0.8	42.4 ± 0.4	65 ± 0.2	68 ± 0.6	0.5 ±0.0
Aedes aegypti	36.7 ±0.9	75.5± 0.4	95.9 ± 0.1	100 ± 0.0	100 ± 0.1	1± 0.1

Each value represent mean of four replicates and ± SE.

Statistical analysis with t test shows that the difference in mortality between Ae. aegypti and Cx. quinquefasciatus larvae was highly significant (p = 0.001).

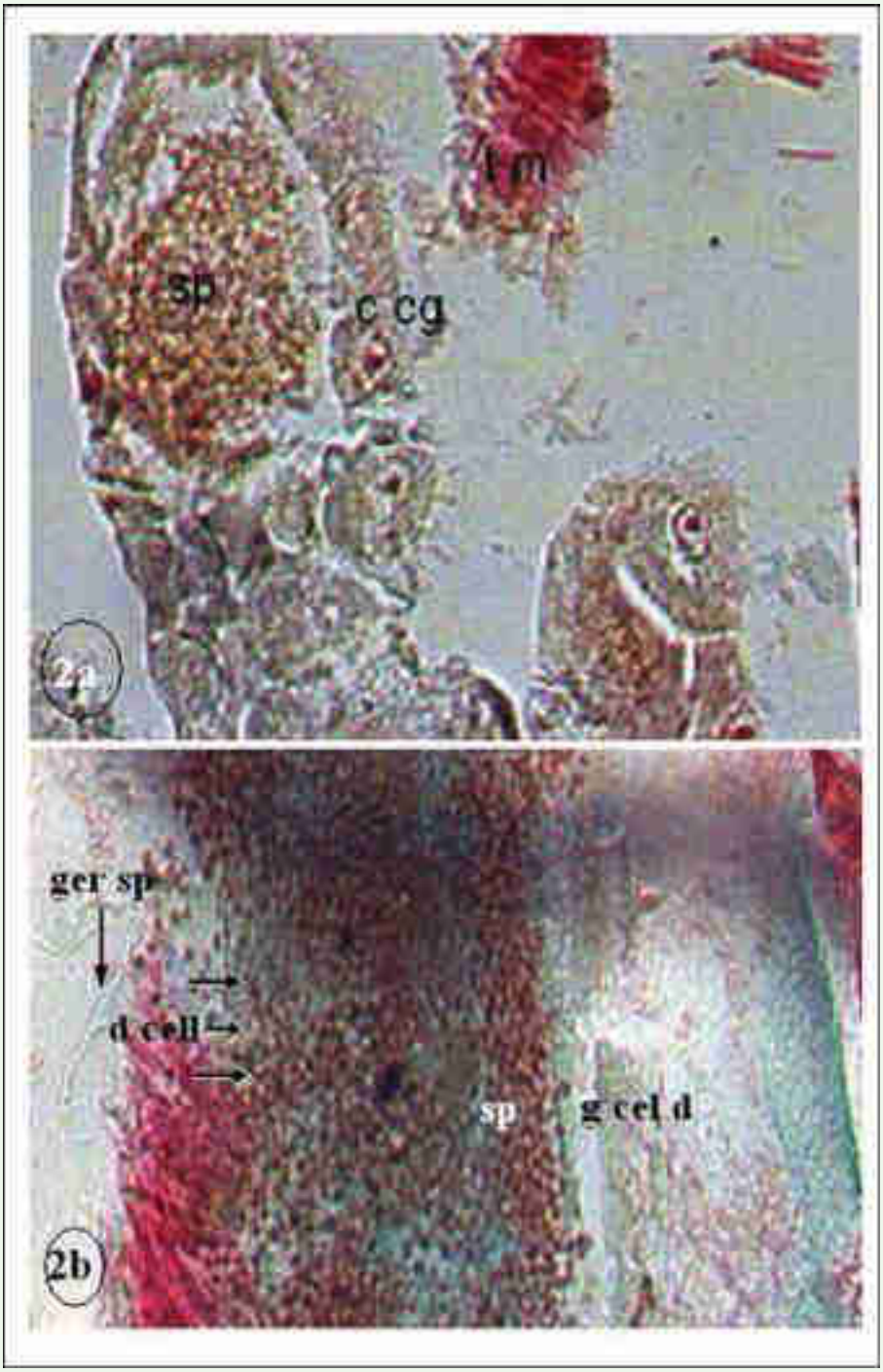

Figure 2. *Anopheles gambiae* gastric cacae (2a) and *Aedes aegypti* gut (2b) after infection by *Aspergillus clavatus.* x 400. c cg = cell of gastric caecum, d cell = disintegrating cells, g cel d= gut cell in disintegration, t m= tissue of muscle.

invasion by *A. clavatus* spores in *An. gambiae* larvae. In the digestive tract for *Ae. aegypti* larvae, it was observed germinating conidia, rupture and disintegrating cells of gut. This has also been reported in previous histological studies (Lacey et al. 1988; Lord and Fukuda 1988; Silva et al. 2004b). According to Crisan (1971) and Lacey et al. (1988), a partial digestion of fungal conidia in the gut may induce a release of toxic substances. Silva et al. (2004b) revealed that rupture and disintegration of cells in the gut on dead larva might be due to the chitinolytic enzymes or others substances produced by the spores. According to Hajek and St. Leger (1994), aggressiveness of entomopathogenic fungus is related to proteolytic, lipolytic and chitinolytic mechanisms that can act after conidial adhesion on the larval cuticle or after invasion of the gut (Crisan 1971; Lacey et al. 1988; Domnas and

Warner 1991; Silva et al. 2004b). *A. clavatus* produces a number of secondary metabolites as tryptoquivaline and tryptoquivalone (Clardy et al. 1975; Buchi et al. 1977); cytochalasin (Demain et al. 1976; Steyn et al. 1982; Lopez-Diaz and Flannigan 1997) and patulin (Varga et al. 2003). The pathological effects noted on the larvae treated with *A. clavatus* in our experiments might be due to these substances.

Light microscopy observations showed that *A. clavatus* conidia produce germ tubes on *Cx. quinquefasciatus* larval cuticle and germinate. Sweeney (1978) showed that with temperatures higher than 30°C, spores of *Culicinomyces* sp. could adhere to the cuticle and invade the gut of *An. amictus* or that of *Cx. fatigans* larvae. This would explain the speed of *A. clavatus* germination on larvae incubated at temperatures ranging between 24 and 26°C.

Cx. quinquefasciatus larvae treated with *A. clavatus* could pupate and produce adults. The resulting adults were collected seven days later and incubated and displayed fungal germination on their abdominal extremities There was no fungal germination on adults that resulted from untreated larvae (control). This suggests that adult mosquitoes that result from treated larvae are likely contaminated at a pre-imaginal stage. Such an observation was also reported in previous studies on adult mosquitoes. Indeed *Ae. albopictus* larvae (Laird et al. 1992) and *Ae. aegypti* larvae in contact with fungus such as *Coelomomyces* could pupate and produce infected adults (Lucarotti and Shoulkamy 2000). According to Laird et al. (1992), infection of adult *Ae. albopictus* by the fungus *Coelomomyces stegomyiae var stegomyiae* could be mortal.

However, in our study, no mortality was recorded for adults reared during 7 days. Lucarotti (1992) found that, on adult mosquitoes, infection by *C. stegomyiae* targets mainly the ovaries, which may explain the germination of *A. clavatus* on the tip of the abdomen.

The laboratory results show that *A. clavatus* isolated from *O. senegalensis*, is virulent against *Ae. aegypti*, *An. gambiae* and *Cx. quinquefasciatus* larvae and could be developed as a biological control agent against mosquitoes. However, further studies are needed for *A. clavatus* strain optimization, and development of better substrates for mass production and practical use. Characterization and application of toxins on mosquito larvae are needed to better understand their rapid killing effects.

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STUDII DE PARAZITOLOGIE / ENTOMOLOGIE

Compatibilité entre *Aspergillus clavatus* (Hyphomycetes) et l'huile de neem (*Azadirachta indica*) contre le moustique vecteur de filarioses *Culex quinquefasciatus* (Say, 1823) (Diptera : Culicidae)

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Résumé

Certaines espèces de champignon appartenant au genre Aspergillus ont montré leur effet larvicide sur les moustiques. Cependant, leur action sur les adultes n'a pas été montrée. Dans nos travaux, nous avons formulé des spores de Aspergillus clavatus (Hyphomycetes) par une huile de neem émulsionnable (Suneem 1%). Dans les conditions du laboratoire, l'application de la formulation Suneem + champignon (S+A) et du Suneem (S) a été effectuée avec une dose de 40 ml/m^2 sur des adultes de Culex quinquefasciatus. Les résultats ont montré que les mortalités cumulées ont été plus fortes avec la formulation qu'avec le Suneem (p= 0,06) ou le témoin (P=0,008). Les mortalités moyennes en 5 jours ont été de 86 ± 1,7 % et 29 ± 1,14 % pour respectivement S+A et S comparées au témoin (3 ± 1,1 %). Par ailleurs, les résultats ont montré que les mortalités sont plus fortes chez les mâles (54 ±1,3 % et 19 ±1,1 %) que chez les femelles (32 ±1,1 % et 10 ± 0,3%) pour respectivement S+A et S. L'infection des adultes traités avec la formulation S+A a été montrée par une forte germination du champignon. Nos travaux montrent donc que le Suneem facilite non seulement l'adhésion des spores sur les adultes de Cx quinquefascitus, mais agit comme adulticide. Il apparait ainsi une possibilité de synergie entre le Suneem et le champignon A. clavatus contre les moustiques. L'effet synergique de ces deux produits sur des larves et des adultes de différentes espèces de moustiques mérite donc d'être étudier.

Mots clefs **:** *Aspergillus clavatus*, *Azadirachta indica*, *Culex quinquefasciatus*, lutte biologique

Introduction

En Afrique, certaines espèces de moustique appartenant aux genres *Anopheles*, *Aedes* et *Culex* sont responsables de la transmission de parasites responsables de maladies (malaria, fièvre jaune, filarioses etc.). Dans la lutte contre les moustiques vecteurs, des études ont montré l'efficacité de produits de neem **(13, 15, 22, 23)** et de champignons **(9, 18, 19, 20, 24).** Certaines espèces de champignons appartenant au genre *Aspergillus* sont connues pour leur effet pathogène contre les larves de moustiques (*Aspergillus parasiticus* **(5)**, *A. flavus* et *A. parasiticus*, **(9)**). Cependant, l'application des spores de champignon contre les moustiques adultes nécessite une formulation pour permettre l'adhésion et la pénétration par la cuticule. Selon Bradley et Britton (1995), les spores de champignons tels que *Beauveria bassiana* et d'autres espèces entomopathogènes sont fortement hydrophobes et difficiles à suspendre dans l'eau. Beaucoup d'applications des mycètes utilisant l'eau, les suspensions détersives, ou les poudres mouillables, emploient les argiles comme aides de suspension ou poudre sèche. Cependant, dans des suspensions en eau, les spores peuvent germer et perdre leur infectiosité après seulement vingt-quatre heures **(4)**. Actuellement, seules certaines espèces de champignons telles que *Metarhizum anisopliae* **(3, 6, 10)** et *Beauveriae bassiana* **(7)** sont utilisées dans des formulations huileuses. La compatibilité entre l'huile de neem et certains champignons entomopathogènes (*M. anisolpliae* et *Beauveriae bassiana*) a été montrée **(7)**. Par contre, la compatibilte entre l'huile de neem et certaines espèces appartenant au genre *Aspergillus* telle que *Aspergillus clavatus* n'a pas été montré.

Notre étude consiste à montrer la compatibilité entre *A. clavatus* et une huile de neem émulsionnable (Suneem1%) contre des adultes males et femelles de moustique vecteur de filariose *Culex quinquefasciatus*.

Matériel et méthodes

Huile de neem (Suneem 1%)

Le Suneem1% est formulée par une industrie chimique Sénégalaise (SENCHIM). C'est une formulation émulsionnable obtenue à partir de l'huile de neem pure enrichie en matière active (m.a.) (Azadirachtine).

Le champignon

Des échantillons du champignon *A. clavatus* isolé à l'Université Cheikh Anta Diop de Dakar et identifié au laboratoire ont été conservés au réfrigérateur. Après culture sur farine de blé stérilisée à 120°C pendant10 mn, les spores sont récupérées et conservées au froid. La teneur en spores (nombre de spores par gramme) a été effectuée sur lame hématimètrique.

Les moustiques

Des larves de *Culex quinquefasciatus* ont été récoltées au niveau des bacs du Département de Biologie Animale (Université Cheikh Anta Diop de Dakar, Sénégal). Elles sont élevées dans des bocaux de dimension 10 x 10 x 7 cm à une humidité relative de 70 à 85% et une température moyenne de 26°C. Elles ont été nourries avec de la chapelure de pain. Après émergence des adultes dans des cages de dimension 50 x 50 cm, nous avons séparé les femelles des males dans deux cages. Tous les adultes ont été nourris avec une solution de sucrose 6% pendant 48 h avant les tests.

Préparation du produit

Dans un Erlenmeyer de 250 ml contenant 100 ml de Suneem 1%, nous avons versé une quantité de 1 g de spores sèches (79 10^9 spores). Le tout est placé sur un agitateur pendant 2h pour avoir un produit mixte S+A (79 10^7 spores/ml et 10 mg m.a./ml).

Pour la solution de pulvérisation nous avons dilué 10 ml de cette solution S+A par 90 ml d'eau distillée dans un tube de 250 ml. La teneur de cette solution est donc 7,9 10^7 spores/ml + 1mg de m.a./ml.

Test sur les moustiques:

Trois séries de traitements ont été faites pour la même dose. Pour chaque série, nous avons utilisé 3 cages cubiques (50 x 50 cm). Dans chaque cage, nous avons mis 50 males et 50 femelles de *Culex quinquefasciatus*. Nous avons pulvérisé la formulation du champignon et l'huile de neem à une dose de 40 ml/m^2 respectivement dans les deux premières cages, la troisième ayant servi de témoin (pas de pulvérisation). Nous avons choisi cette dose en spores en nous référant sur la gamme de doses choisies par Scholte *et al*., 2003 **(18)**. Les tests avec l'huile de neem formulée (Suneem) sont réalisés pour voir son effet sur les adultes.

La pulvérisation a été faite à travers les mailles de la toile moustiquaire. Les moustiques seront donc infectés directement au moment de la pulvérisation ou au moment du repos sur la toile moustiquaire. Les constats de mortalité et du nombre de survie (mâle et femelle) sont effectués toutes les 24h (pendant 5 jours). Ces deux constats sont faits pour vérifier l'effectif total (50 males et 50 femelles). Pour vérifier l'infection des adultes par le champignon, les moustiques morts sont placés dans des boites de Pétri avec papier filtre imbibé d'eau distillée. Les observations ont été effectuées à la loupe 7 jours après incubation.

Analyse des données

Les pourcentages de survie male et femelle sont utilisées pour l'analyse de survie avec le logiciel Statview. Les mortalités sont corrigées avec la formule d'Abbott **(1)**. Les mortalités cumulées des adultes traités avec la formulation S+A et S sont utilisées pour effectuer le test t de Student.

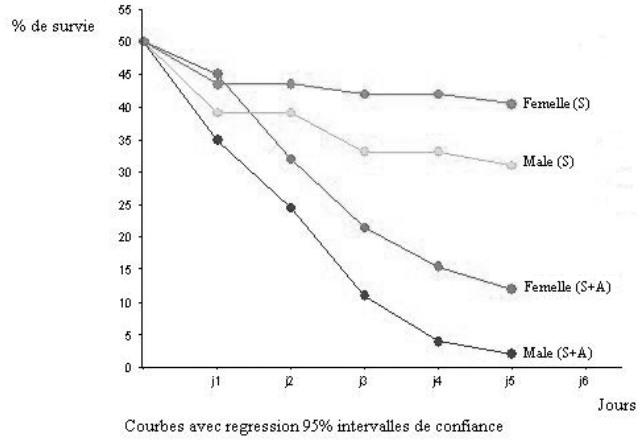
Résultats

Tout juste après pulvérisation des deux produits S+A et S sur les moustiques adultes, nous avons constaté une chute rapide d'environ 90% des adultes. Quelques minutes après (30 mn), presque 95% avaient repris leur vol et se trouvaient sur les parois de la cage. Vingt quatre heures après pulvérisation, nous avons commencé à constater les mortalités. Les résultats ont montré que les courbes de régression de survie des adultes traités aux deux produits sont plus basses chez les males que chez les femelles (**Figure 1**). Cependant, les mortalités cumulées (**Figure 2**) ont été plus importantes avec la formulation (S+A) qu'avec le Suneem seul (S) (p= 0,06) ou le témoin (P= 0,008). L'incubation des adultes morts pendant 7 jours sur papier filtre humide montre une forte germination (>95%) du champignon *A. clavatus* (**Figure 3**). Par contre, aucune germination n'a été observée sur les adultes non traités au champignon.

Tableau 1 - Pourcentage de mortalités cumulées des adultes males et femelles de Culex quinquefasciatus traités au S+A ou S (A= Aspergillus clavatus, S= Suneem, T= Témoin).

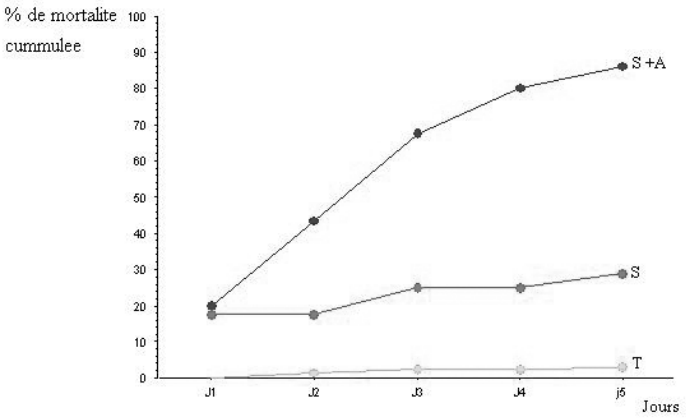
	S+A			S			T
Jours	Male	Femelle	Total	Male	Femelle	Total	
J1	15 ± 0,7	5 ± 0,4	20 ± 0,11	11 ± 0,1	6,5 ± 1,5	17,5 ± 1,6	0±0,0
J2	25,5 ± 1,3	18 ± 0,01	43,5 ± 1,3	12 ± 0,5	6,5 ± 0,4	18,5 ± 0,9	1,5 ± 0,4
J3	39 ± 0,2	28,5 ± 0,1	67,5 ± 0,3	17 ± 0,4	8 ± 1,3	25 ± 1,7	2,5±0.3
J4	46 ± 0,7	34 ± 0,6	80 ± 1,3	17 ± 0,4	8 ± 0,09	25 ± 0,5	2,5±1,2
J5	54±1,3	32 ±0,4	86±1,7	19±1,1	10±0,3	29±1,14	3 ±1,1

Figure 1 - Pourcentage de survie des adultes de Culex quinquefasciatus traités aux produits S+A ou S.



Pourcentage de survie des adultes male et femelle de Culex quinquefasciatus traites aux produits S ou (S+A). S= Suneem, A = Aspergillus clavatus.

Figure 2 - Pourcentage de mortalités cumulées des adultes de Culex quinquefasciatus traités aux produits S+A ou S.



Mortalites cumulees des adultes de Culex quinquefasciatus traites aux produits S ou S+A. S= Suneem, A= Aspergillus clavatus.

Figure 3 - Culex quinquefasciatus mâle non infecté et infecté par Aspergillus clavatus 7 jours après incubation. Ab. = Abdomen, Ai= Aile, An. = Antenne, T. = Tête, Th. = Thorax.

Discussion

Selon Kirschbaum (1985) **(11)**, les champignons pathogènes aux insectes et leurs dérivés peuvent être une approche prometteuse pour la lutte biologique contre les moustiques. Plusieurs espèces de champignons appartenant au genre *Aspergillus* telles que *Aspergillus parasiticus*, *A. flavus* ont montré leur effet pathogène contre les larves de moustique. Cependant, l'effet pathogène d'une espèce de champignon appartenant au genre *Aspergillus* n'a pas été montré sur les moustiques adultes.

Dans nos travaux, l'utilisation de *Aspergillus clavatus* formulé avec une huile émulsionnable (Suneem) a montré un effet adulticide contre *Culex quinquefasciatus*. Cependant, les mortalités diffèrent selon le sexe du moustique adulte. Comparés aux travaux de Scholte *et al*. 2003 **(18)**, *A. clavatus* a les mêmes effets que *Metarhizium anisopliae* contre les adultes de *Cx quinquefasciatus*. En effet, nos résultats ont montré que 5 jours après application de la formulation de *A. clavatus*, les mortalités observées chez les adultes mâles sont plus importantes (54 ±1,3 %) que celles observées chez les femelles (32 ±1,1 %). Cependant, Scholte *et al*. 2003 **(18)** ont obtenu avec *M. anisopliae* un TL_{50} de 5 jours chez les adultes avec $1,6 \times 10^{10}$ spores/m^2 alors que nous avons obtenu avec *A. clavatus* une mortalité de 86,5 ±0,71 % pour une dose de $3,16\ 10^9$ spores/m^2. Cette différence de mortalité pourrait être expliquée par la sensibilité de *Cx quinquefasciatus* par rapport aux deux espèces de champignon ou par l'huile de formulation du champignon. En effet, Scholte *et al*. 2003 **(18)** ont utilisé l'huile de tournesol, alors que dans nos travaux nous avons utilisé le Suneem contre la même espèce de moustique. Par ailleurs, des travaux ont montré que le Suneem a un effet larvicide sur *Anopheles gambiae* **(14)**. Des travaux ont montre que les produits de neem ont un effet de blocage sur l'oogenèse du moustique *Anopheles stephensi* **(12)** et un effet répulsif **(16, 21)**. L'utilisation du Suneem expliquerait donc les fortes mortalités que nous avons obtenues en 5 jours (86, ± 0,71 %) avec une dose de $3,16\ 10^9$ spores/m^2. Néanmoins, cette mortalité obtenue sur *Cx quinquefasciatus* est proche de celle trouvée par Scholte *et al*., 2007 **(20)** sur *Aedes aegypti* (87,1 ± 2,67 %) et sur *Aedes albopictus* (89,3 ± 2,2 %) avec l'utilisation du *M. anisopliae* mais à une dose plus forte que la notre ($1,6\ 10^{10}$ spores/m^2). Dans leurs travaux, ils ont aussi montré une différence de sensibilité entre les males et les femelles vis-à-vis du champignon.

Les fortes mortalités observées avec le champignon formulé pourraient donc être expliquées par un effet de synergie entre *A. clavatus* et l'azadirachtine contenue dans l'huile de neem. La germination du champignon sur les adultes montre la compatibilité entre le Suneem et *A. clavatus*. Des études ont montré des cas de synergisme entre différents biopesticides contre les moustiques **(8)**. Cela montre donc la possibilité de combiner différents biopesticides compatibles pouvant agir en synergie. Cette nouvelle approche pourrait être une alternative à l'utilisation de certains insecticides chimiques pour une lutte efficace contre les moustiques vecteurs.

Nos travaux ont montré la compatibilité de l'huile de neem (Suneem) avec *A. clavatus*, leur action adulticide sur *Cx. quinquefasciatus* et une possibilité de synergisme. Cependant, l'azadirahtine contenue dans l'huile de neem est sensible aux

rayonnements ultraviolets et a une durée de vie déterminée **(17)**. Selon Bradley et Britton (1995) **(4)**, les spores sont mieux protégées par les formulations huileuses contre les rayons ultraviolets mais avec une durée de vie de 40 jours. Nos travaux n'ont pas montré la durée de la compatibilité entre *A. clavatus* et le Suneem. Il est donc nécessaire d'étudier d'une part l'effet synergique et la durée de la compatibilité entre l'huile de neem formulée et *Aspergillus clavatus* ; et d'autre part l'effet de cette formulation sur les larves et adultes d'autres espèces de moustique vecteurs de parasites.

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Rezumat

Unele specii de ciuperci din genul Aspergillus şi-au arătat efectul larvicid asupra ţânţarilor. Totuşi, acţiunea lor asupra adulţilor nu a fost demonstrată. În studiul nostru am folosit spori de Aspergillus clavatus (Hyphomycetes) în ulei de neem emulsionabil (Suneem 1%). În laborator, aplicarea formulării Suneem + ciupercă (S+A) şi Suneem (S) s-a făcut în doză de 40 ml/m^2 pe adulţi de Culex quinquefasciatus. Rezultatele au arătat că mortalităţile cumulate au fost mai mari cu formularea combinată decât cu Suneem (p= 0,06) sau martor (P=0,008). Mortalităţile medii după 5 zile au fost de 86 ± 1,7 % şi respectiv 29 ± 1,14 % pentru S+A şi respectiv S, comparate cu martorul (3 ± 1,1 %). În plus, rezultatele au arătat că mortalităţile sunt mai ridicate în rândul masculilor (54 ±1,3 % şi 19 ±1,1 %) decât al femelelor (32 ±1,1 % şi 10 ± 0,3%) pentru S+A şi respectiv S. Infecţia adulţilor trataţi cu formula S+A a fost demonstrată printr-o intensă germinare a ciupercii. Studiul nostru arată deci că Suneem nu facilitează doar adeziunea sporilor la adulţii de Cx quinquefascitus, ci acţionează şi ca adulticid. Există posibilitatea unei sinergii între Suneem şi ciuperca A. clavatus în acţiunea împotriva ţânţarilor. Efectul sinergic al acestor două produse asupra larvelor şi a adulţilor din diferite specii de ţânţari merită deci să fie studiat.

Cuvinte cheie**:** *Aspergillus clavatus*, *Azadirachta indica*, *Culex quinquefasciatus*, *luptă biologică*.

Afrique SCIENCE *02(2) (2006) 212 - 225*
ISSN 1813-548X

Etude comparative de deux produits de neem (huile et poudre) sur les stades préimaginaux du moustique *Culex quinquefasciatus* (Diptera : Culicidae)

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(Reçu le 14 Décembre 2005, accepté le 17 Mai 2006)

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Résumé

Des produits de neem (*Azadirachta indica*) (huile de neem formulée 1 % et poudre de neem 0,3 %) fabriqués par une industrie sénégalaise (SENCHIM), ont été appliqués sur des larves et nymphes du moustique (*Culex quinquefasciatus).* Les doses ont été de 0,02 à 0,038 % (v/v) et de 0,2 à 2 % (p/v) respectivement pour l'huile de neem formulée et la poudre de neem.

Les résultats dans les conditions du laboratoire ont montré que la poudre de neem était plus toxique au stade larvaire (mortalités entre 86,1 et 100 %) qu'au stade nymphal (entre 14,5 et 95,9 % d'adultes envolés). Cependant, l'huile de neem formulée a été efficace aussi bien au stade larvaire (mortalité entre 52,1 et 80 %) qu'au stade nymphal (entre 0 et 14 % d'adultes envolés). L'étude histopathologique a montré que les produits agissent au niveau du système digestif des larves après ingestion alors qu'au niveau des nymphes seul l'effet contact est observé. L'huile de neem formulée 1 % apparaît plus efficace que la poudre de neem 0,3 % pour le contrôle des moustiques en milieu aquatique.

Mots-clés : *neem, Azadirachta indica, stade préimaginal, Culex quinquefasciatus.*

Abstract

Comparative study of two neem products (oil and powder) on preimaginal stages of *Culex quinquefasciatus* mosquito (Diptera: Culicidae)

Neem (*Azadirachta indica*) products (neem oil formulated 1 % and neem powder 0.3 %), manufactured by a Senegalese industry (SENCHIM), were applied to mosquito (*Culex quinquefasciatus*) larvae and nymphs. The doses products were from 0.02 to 0.038 %

(neem oil) and 0.2 to 2 % (powder). In laboratory conditions, results showed that neem powder was more toxic at larval stage (mortalities between 86.1 and 100 %) than nymphal stage (between 14.5 and 95.9 % adults flow away). Neem oil formulated was effective as well as larval stage (mortality between 52.1 and 80 %) than nymphal stage (between 0 and 14 % adults flown away). The histopathological study reveals that neem products act upon larval digestive system after ingestion, whereas for nymphs only contact effects are showed. Neem oil formulated 1 % appears more effective than neem powder 0.3 % for mosquitoes control in aquatic environment.

Keywords : *Neem, Azadirachta indica, preimaginal stage, Culex quinquefasciatus.*

1. Introduction

Malgré leur efficacité dans la lutte contre les insectes et plus particulièrement contre les moustiques, l'utilisation des insecticides chimiques de synthèse présente des inconvénients par l'apparition des effets indésirables [1-3]. L'utilisation des produits naturels devient alors une perspective de recherche et fait l'objet de plusieurs investigations. Dans le cadre de la recherche d'insecticides naturels, le neem figure parmi les plantes les plus utilisées. Le neem (*Azadirachta indica)* est un arbre d'origine Asiatique que l'on trouve souvent dans les régions arides et semi-arides. Au niveau de cette plante, la quantité d'huile et des principaux triterpenoïdes varient périodiquement au cours de l'année [4]. Les amandes de neem sont riches en triterpènes et en huile.

Différents produits sont formulés à partir de l'huile de neem (Neemix, Margosan-O, etc.) et utilisés contre les insectes. Son caractère insecticide a été étudié sur les larves de certains moustiques tels que *Culex* sp. [5], *Anopheles* sp [6], *Aedes aegypti* [7]. La principale matière active est l'azadirachtine. La teneur en matière active est plus élevée dans les graines que dans le reste de la plante [6]. Après pressage des amandes de neem, la totalité de la matière active reste dans les tourteaux alors que l'huile n'en possède qu'une faible teneur. La poudre est généralement utilisée par les jardiniers pour la protection des cultures. Le neem a des caractères antiappétant, répulsif et inhibiteur de la croissance des insectes. Des extraits aqueux de neem ont montré des effets néfastes sur la fertilité, la fécondité et le développement post-embryonnaire de certains diptères [8]. Les travaux de [9] ont montré que l'huile des amandes de neem est plus efficace contre l'oviposition et contre l'émergence des adultes de l'insecte *Callosobruchus maculatus* que la poudre de neem.

En milieu urbain et périurbain, la prolifération des moustiques en particulier celle du *Culex quinquefasciatus* est observée en période hivernale et post-hivernale. L'utilisation des produits de neem (huile et poudre) contre les larves de moustiques pourrait réduire

cette prolifération. Cependant, pour les stades préimaginaux des moustiques, la phase nymphale est plus courte que la phase larvaire. De ce fait, l'efficacité et l'action de ces deux produits peuvent différer selon le stade préimaginal. Il est donc nécessaire de faire une étude comparative de ces deux produits sur les stades préimaginaux des moustiques.

La présente étude consiste à :

- préciser le stade le plus sensible pour chaque produit,
- montrer à quel niveau agissent les deux produits pour chaque stade,
- proposer le produit le plus efficace pour lutter contre la prolifération des moustiques.

2. Matériel et méthodes

2-1. Matériel

2-1-a. Matériel animal

Des œufs du moustique *Culex quinquefasciastus* sont récoltés quotidiennement au niveau des bacs artificiels du département de Biologie Animale. Les œufs sont mis à éclosion dans des bacs contenant de l'eau du robinet. Après éclosion, les larves de première génération sont nourries avec de la poudre de pain en prenant le soin de changer régulièrement l'eau d'élevage. Ainsi, nous avons obtenu successivement des larves de stades L1, L2, L3, L4 et des nymphes.

2-1-b. Matériel végétal

Le matériel végétal nous a été fourni par la SENCHIM, une industrie chimique Sénégalaise.

2-2. Méthodes

2-2-a. Traitement avec l'huile de neem formulée

Pour chaque traitement, nous avons utilisé 11 bocaux de 10 cm de côté et 7 cm de hauteur contenant chacun 500 mL d'eau. Pour les larves, nous avons mis dans chaque bocal 100 larves dont 25 individus pour chaque stade larvaire et de la poudre de pain comme nourriture. Pour les nymphes, le même nombre d'individus est utilisé.
Le volume en huile de neem formulée varie de 0,02 % à 0,038 % avec des pas de 0,002 %.
Nous avons fait des constats de mortalité toutes les 24 h en faisant chaque jour le cumul des mortalités.

2-2-b. Traitement avec la poudre de neem

La quantité de poudre varie de 0,2 % à 2 % avec des pas de 0,2 %. Ces doses sont choisies suite à des tests antérieurs que nous avons effectués au laboratoire.
Le même nombre d'individus est utilisé aussi bien pour les larves que pour les nymphes.
Nous avons fait des constats de mortalité toutes les 24 h en faisant chaque jour le cumul des mortalités.
Pour les traitements, la température moyenne pour les traitements était de 26°C ± 0,55 et le taux d'humidité moyenne était de 88 % ± 0,14.

2-2-c. Analyse statistique

Nous avons effectué deux séries de traitements et les valeurs données représentent la moyenne des deux séries. Les mortalités corrigées sont calculées en appliquant la formule d'Abbott :

$$\% m = (NL M - NLMT) X 100 / (NTL - NLMT) \quad (1)$$

%m = pourcentage de mortalité
NLM = nombre de larves mortes
NLMT = nombre de larves mortes dans le témoin
NTL = nombre total de larves

Pour les nymphes, nous avons considéré en même temps la mortalité des nymphes et des adultes issus des nymphes traitées. Pour connaître le pourcentage d'adultes envolés, nous avons fait la différence entre le nombre de nymphes traitées et le nombre d'individus morts.

Le pourcentage d'adultes envolés est obtenu en faisant cette différence :

$$\% A.E. = 1 - \% (N.M. + A.M.) \quad (2)$$

% A.E. = Pourcentage d'adultes envolés
N.M. = Nymphes Mortes
A.M. = Adultes Morts

Le logiciel Excel est utilisé pour le calcul des moyennes de mortalité pour chaque stade.
Pour chaque produit, nous avons utilisé le test *Khi-deux* (X^2) pour voir si l'efficacité des produits entre les larves et les nymphes est significativement différente ou non.

2-2-d. Etude histologique

Après traitement, les larves et nymphes moribondes sont fixées au *carnoy2* pendant 72 h. Les larves sont ensuite soumises successivement à une déshydratation, imprégnation et inclusion. Après confection et la taille des blocs, les coupes de 7 µm d'épaisseur faites au

microtome sont colorées par la méthode du trichome de Masson. Les observations sont faites au microscope photonique Motic relié à un micro-ordinateur où les photos sont enregistrées sous format JPEG.

3. Résultats

3-1. Effets insecticides des produits de neem sur les larves de *C. quinquefasciatus*

3-1-a. Cas de l'huile de neem formulée

Tableau 1 : *Pourcentages de mortalités des larves de Culex quinquefasciatus traitées avec l'huile de neem formulée 1 %*

Jours	Pourcentages en huile de neem formulée (1 %)										Témoin
	0,02	0,022	0,024	0,026	0,028	0,03	0,032	0,034	0036	0,038	
J1	24,5	27,5	24,5	22	25	16	22	24,5	25	41,5	2
J2	32	43	41	47	40	52	43,5	43	55,5	58,5	3
J3	37,5	51	52,5	60	51	58,5	56	54	67	62	8
J4	43	51,5	54	66,5	56,5	61,5	57,5	58	72	69	8
J5	48,5	54	58,5	74,5	60	67,5	59,5	60	74,5	70,5	9
J6	51	56	62,5	77,5	64,5	68,5	62	61,5	77	74	10
J7	52,5	62,5	67,5	82,5	68,5	71,5	63,5	63,5	80	77,5	11

Le ***Tableau 1*** montre une mortalité croissante des larves de *Culex quinquefasciatus* au cours du temps. Ce tableau montre des mortalités allant de 52,5 à 80 % sept jours après traitement par rapport au témoin (11 % de mortalité). Cependant, nous n'avons pas obtenu 100 % de mortalité. Une semaine après test, la moyenne des mortalités est de 68,95 %.

3-1-b. Cas de la poudre de neem

Tableau 2 : *pourcentages de mortalités des larves de Culex quinquefasciatus traitées avec la poudre de neem 0,3 %*

jours	Pourcentages en poudre de neem (0,3 %)										Témoin
	0,2	0,4	0,6	0,8	1	1,2	1,4	1,6	1,8	2	
J1	1,5	4,5	4,5	4,5	8	13,5	13	11	10,5	16	0
J2	5,5	8	16	22,5	27,5	41,5	53	65,5	74,5	74	0
J3	12	30	39,5	51	50	69	69,5	77	86	79	0
J4	37,6	42,7	74,8	64,7	65,7	76,2	78,3	98,4	98,4	96,4	0,5
J5	62,9	68,5	83,7	82,7	94,8	95,4	93,3	100	100	100	1,5
J6	68,8	80	83,8	85,7	95,8	96,4	96,9	100	100	100	2
J7	86,1	87,1	95,8	90,2	96,8	96,8	98,9	100	100	100	2,5

Le ***Tableau 2*** montre une mortalité croissante des larves de *Culex quinquefasciatus* au cours du temps mais aussi avec les doses (0,2 à 2 %). Les doses létales 50 (1,4 %) et 100 (2,6 %) sont obtenues respectivement en 2 et 5 jours. Une semaine après, la moyenne des mortalités est de 95,17 %.

3-2. Effets insecticides des produits de neem sur les nymphes de *C. quinquefasciatus*

3-2-a. Cas de l'huile de neem formulée

Tableau 3 : *pourcentages d'adultes envolés en fonction de la quantité d'huile de après traitement des nymphes de Culex quinquefasciatus*

Jours	Pourcentages en huile de neem formulée (1 %)										Témoin
	0,02	0,022	0,024	0,026	0,028	0,03	0,032	0,034	0,036	0,038	
J2	0,6	2,2	7,9	14	12,9	8,1	0	0	0	0	84,5

Le ***Tableau 3*** montre une nette diminution des pourcentages de moustiques adultes envolés (entre 0 et 14 %) en fonction des doses en huile de neem par rapport au témoin (84,5 %). La moyenne des mortalités (m_1) est de 95,43 % par rapport au témoin

(13,5 %). A partir de la dose 0,032 %, aucun adulte ne s'est envolé. La majeure partie des individus traités est morte 2 jours après.

3-2-b. Cas de la poudre de neem

Tableau 4 : *pourcentages d'adultes envolés en fonction de la quantité poudre de neem après traitement des nymphes de Culex quinquefasciatus*

Jours	Pourcentages en poudre de neem (0,3 %)										Témoin
	0,2	0,4	0,6	0,8	1	1,2	1,4	1,6	1,8	2	
J2	95,9	87,7	86,6	80,5	81,5	53,7	40,3	20,7	25,8	14,5	97

Le ***Tableau 4*** montre une diminution des pourcentages de moustiques adultes envolés (entre 14,5 et 95,5 %) en fonction de la teneur en poudre par rapport au témoin (97 %). Cependant cette diminution est plus faible par rapport à l'huile de neem formulée. La moyenne des mortalités (m_2) est de 41,28 % et donc la majeure partie des individus est passée en adulte et s'est envolée.

3-3. Comparaison de l'efficacité de chaque produit sur les deux stades préimaginaux

3-3-a. Avec l'huile de neem formulée

L'application du test *Khi-deux* montre que l'efficacité de l'huile de neem formulée sur les larves et les nymphes n'est pas significativement différente (ddl $=$ 9 ; $\alpha = 5$ % ; X^2 tabulé $=$3,325 ; X^2 calculé $=$ 1,133).

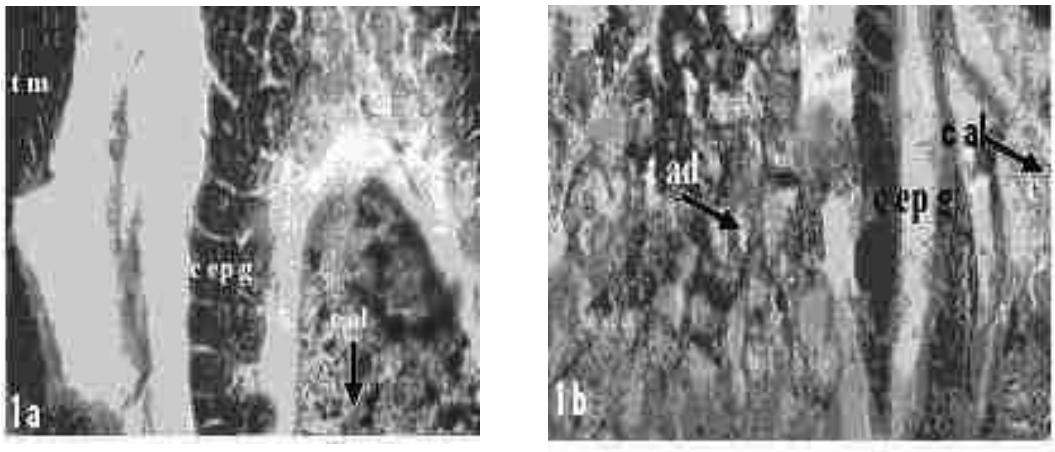
3-3-b. Avec la poudre de neem

L'application du test Khi-deux montre que l'efficacité de la poudre de neem sur les larves et les nymphes est significativement différente (ddl $=$ 9 ; $\alpha = 5$ % ; X^2 tabulé $=$ 3,325 ; X^2 calculé $=$ 8,59).

3-4. Etude histologique des larves et nymphes traitées aux produits de nem

3-4-a. Sur les larves

*** Larves non traitées**



Figures 1a & 1b : *Coupes histologiques des larves de Culex quinquefasciatus non traitées (témoin) (G= 400)*

La coupe longitudinale passant au niveau du thorax des larves de Culex quinquefasciatus non traitées montre la présence de tissus intacts (tissu adipeux (t ad), tissu musculaire (t m). La colonne alimentaire (c al) est présente et est entourée par les cellules épithéliales gastriques (c ep g).

*** Larves traitées avec l'huile de neem formulée**

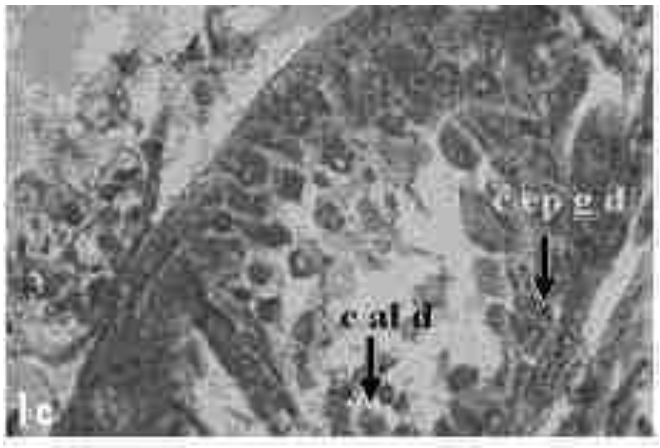


Figure 1c : *Coupe histologique d'une larve de Culex quinquefasciatus traitée à l'huile de neem formulée (G= x 400)*

La coupe longitudinale au niveau antérieure des larves de Culex quinquefasciatus traitées à l'huile de neem formulée (1c) montre, des cellules épithéliales gastriques détruites (c ep g d) envahissant la lumière du caecum, une colonne alimentaire désorganisé (c al d) et un tissu adipeux absent par rapport au témoin ***(Figures 1a et 1b)***.

*** Larves traitées avec la poudre de neem**

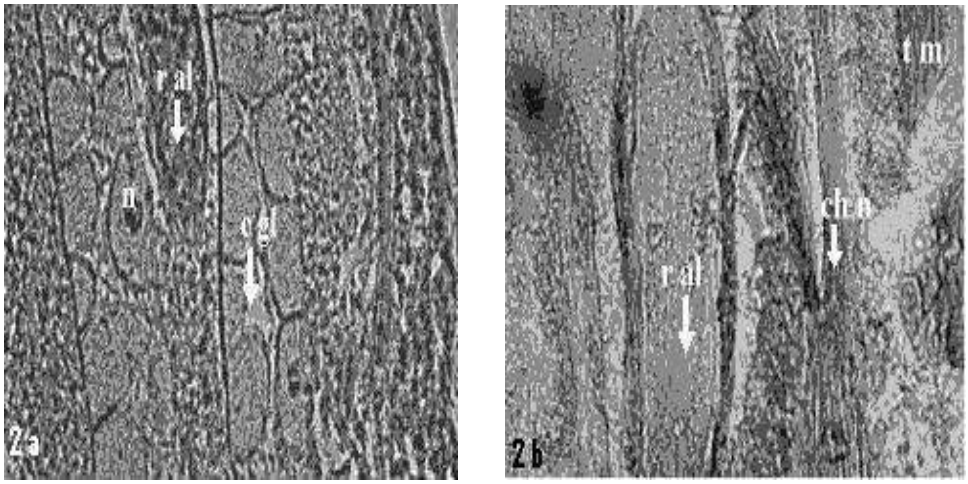
Figure 1d : *Coupe histologique d'une larve de Culex quinquefasciatus traitée à la poudre de neem (G = x 400)*

La coupe longitudinale au niveau antérieure des larves de Culex quinquefasciatus traitées à la poudre de neem montre une destruction des cellules épithéliales gastriques (c ep g) et une désorganisation de la colonne alimentaire (c al d). Cependant, la désorganisation des cellules gastriques est moins importante par rapport à celle des larves traitées à l'huile.

3-4-b. Sur les nymphes

*** Nymphes non traitées (témoin)**

Sur les ***Figures 2a et 2b***, nous pouvons noter la présence des cellules glandulaires (c gl), de la chaîne nerveuse (ch n), du tissu musculaire (t m) et des réserves alimentaires (r al) en bon état.



Figures 2a et 2b : *Coupes histologiques des nymphes de Culex quinquefasciatus non traitées (témoin) (G = x 400)*

*** Nymphes traitées avec l'huile de neem formulée**

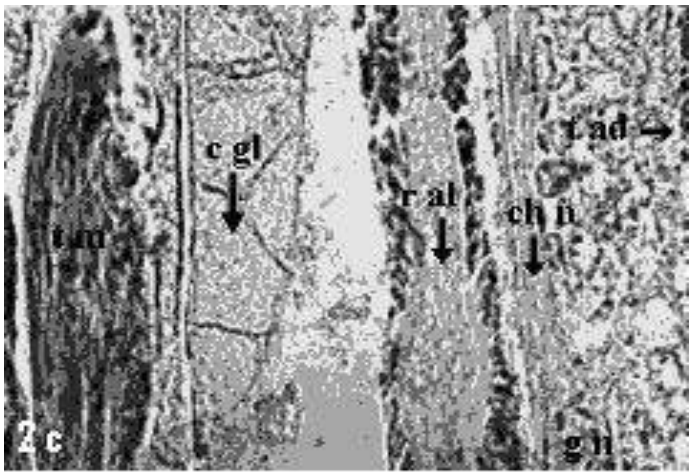


Figure 2c : *Coupe histologique d'une nymphe de Culex quinquefasciatus traitée à l'huile de neem formulée (G = x 400).*

Sur cette ***Figure 2c,*** nous pouvons noter que les organes observés à savoir les cellules glandulaires (c gl), la chaîne nerveuse (ch n), le tissu musculaire (t m), le tissu adipeux (t ad) et les réserves alimentaires (r al), Cependant, nous pouvons noter un début de dégradation des structures par rapport au témoin ***(Figures 2a et 2b)***.

*** Nymphes traitées avec la poudre de neem**

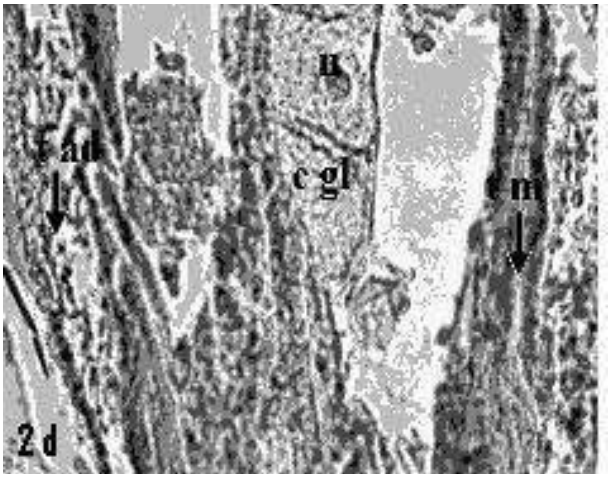


Figure 2d : *Coupe histologique d'une nymphe de Culex quinquefasciatus traitée à la poudre de neem (G= 400)*

Sur la ***Figure 2d,*** nous pouvons noter la présence du tissu adipeux (t ad), des cellules glandulaires (c gl) avec leur noyau (n) et du tissu musculaire (t m). Cependant, nous pouvons noter un début de dégradation des structures par rapport au témoin ***(Figures 2a et 2b)***

4. Discussion

De nombreux travaux ont montré l'efficacité de l'huile de neem sur les insectes [5-7,10]. Cependant, il faut noter qu'en plus de l'action de la matière active, l'huile de neem pure tue les larves de moustique par asphyxie en les empêchant de respirer à la surface de l'eau.

La formulation de l'huile de neem pure en y ajoutant des sulfactants lui permet d'être miscible avec l'eau. Les travaux de [11] ont montré une mortalité de 51,6 % des larves de *Culex quinquefasciatus* en un jour avec 5 % d'huile de neem émulsionnable. Avec l'huile de neem formulée 1 %, nous avons obtenu pour une dose de 0.03 % (soit 3 mg/L en azadirachtine) 50 % de mortalité en 2 jours ***(Tableau 1)***.

Selon [12], la moitié de la durée de vie de l'azadirachtine est de 282 h à 25°C et celle-ci décroît si la température augmente. Nous avons effectué nos travaux dans des conditions de température comprises entre 24 et 28,5°c durant 7 jours.

Les résultats montrent l'effet toxique des produits de neem sur le développement des

larves et nymphes de moustique. Cependant, l'huile et la poudre ne se comportent pas de la même manière. L'huile de neem formulée est aussi efficace contre les larves que contre les nymphes. Ce qui n'est pas le cas pour la poudre de neem. En effet, la matière active (azadirachtine) qui se trouve dans la poudre (solide) est libérée lentement dans l'eau alors que la formulation de l'huile facilite la libération et la diffusion de la matière active. Les fortes mortalités des larves observées (100 %) avec l'application de la poudre de neem ***(Tableau 2)*** sont dues au fait que les doses choisies sont plus fortes que celles de l'huile de neem formulées. Au bout de 2 jours, la majeure partie de la matière active est passée du solide (poudre) vers le liquide (eau). La durée des quatre stades larvaires (L1, L2, L3 et L4) étant plus longue, la matière active peut donc agir avant que les larves ne puissent passer en nymphes.

Les travaux de [13] ont montré que l'application de la poudre de neem à 500 kg/ha ou 50 g/m² a entraîné une mortalité des larves de *Culex quinquefasciatus* à 90 % dans des champs de riz. Considérant nos bocaux ayant une surface de 0,01 m² (10 X 10 cm), nous pouvons faire une correspondance de cette dose qui serait à 0,5 g pour 0,01 m². Dans nos travaux, cette dose correspond à celle ayant donné une mortalité située entre 87 et 95 % en 7 jours. Cette similitude montre bien l'efficacité de la poudre de neem sur les larves de moustiques aussi bien au laboratoire qu'en milieu ambiant.

Le stade nymphal des moustiques dure entre 2 et 3 jours, ce qui fait que les nymphes traitées avec la poudre de neem auront le temps de passer en adulte et de s'envoler avant que le produit n'agisse. Avec l'huile, la mortalité des individus (nymphes et adultes émergés) est plus importante (m_1= 95,43 %) qu'avec la poudre de neem (m_2= 42,28 %). En effet, lors de nos travaux, nous avons constaté que certaines nymphes traitées à l'huile de neem formulée sont mortes avant la nymphose, d'autres au moment de la nymphose (effet contact). Nous avons pu constater que certains adultes issus des nymphes traitées à l'huile de neem n'ont pas pu s'envoler. Ce qui serait du à la nature huileuse qui empêche les moustiques de s'envoler après émergence.

Avec la poudre de neem, la mortalité des nymphes est observée pratiquement au moment de la nymphose (effet contact du produit). Sur le plan physiologique, les nymphes ne se nourrissent pas mais utilisent des réserves nutritives du stade larvaire. Chez les larves, les produits appliqués agissent après ingestion (destruction des cellules gastriques) et par effet contact, alors que chez les nymphes, seul l'effet contact se manifeste. En effet, puisque les nymphes ne se nourrissent pas, les produits ne peuvent donc pas être ingérés. Contrairement aux larves qui en s'alimentant ingèrent en même temps le produit appliqué. Le mode d'action de ces produits sur les cellules gastriques est identique à celui décrit par [14]. L'application de la poudre de neem ne peut empêcher aux nymphes d'évoluer en adulte que si la dose est forte. L'utilisation de l'huile de neem formulée en milieu aquatique est donc plus sûre pour empêcher la

prolifération des moustiques. Néanmoins, la poudre reste toujours efficace contre les larves du moustique *Culex quinquefasciatus.* L'huile de neem formulée et la poudre de neem agissent de la même manière au niveau des larves (destruction des cellules gastriques) mais différemment au niveau des nymphes (effets contacts au moment et après la nymphose).

5. Conclusion

Les résultats de notre étude nous ont permis de dire que l'huile de neem formulée est aussi efficace sur les larves que sur les nymphes. La poudre par contre n'est efficace que sur les larves.
L'huile de neem formulée et la poudre de neem agissent par voie intestinale et par contact sur les larves, alors qu'elles n'agissent que par contact sur les nymphes.
A l'issue de cette étude, nous pouvons dire que l'huile de neem formulée est plus efficace que la poudre de neem sur les stades préimaginaux des moustiques.

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EFFETS LARVICIDES DES PRODUITS DE NEEM (HUILE DE NEEM PURE ET NEEMIX) COMPARES A DEUX INSECTICIDES CHIMIQUES DE SYNTHESE (LA DELTAMETHRINE ET LE FENITROTHION) SUR LES LARVES DU MOUSTIQUE *CULEX QUINQUEFASCIATUS* (DIPTERA : CULICIDAE)

LARVICIDAL EFFECTS OF NEEM PRODUCTS (NEEM OIL AND NEEMIX), COMPARED TO CHEMICAL SYNTHETICS INSECTICIDES (DELTAMETHRIN AND FENITROTHION) ON THE MOSQUITO LARVAE *CULEX QUINQUEFASCIATUS* DIPTERA : CULICIDAE)

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Résumé :
Suite aux problèmes posés par certains insecticides de synthèse, la Science est à la recherche d'autres produits naturels à caractère insecticide. Pour cela, des produits à base d'azadirachtine sont formulés par diverses industries chimiques. L'huile de neem pure et le Neemix formulés par une industrie sénégalaise (SENCHIM) ont montré une forte toxicité sur les larves de *Culex quinquefasciatus*. Les travaux effectués dans les conditions du laboratoire ont montré que la DL100 (Dose létale 100) de l'huile de neem pure en 24 h était de 9% alors qu'elle était de 0,028% pour le Neemix. Notre étude a révélé de façon inattendue que, le solvant utilisé (Solvesso 100) pour la formulation du Neemix était très toxique pour les larves.
Par ailleurs, la comparaison de ces produits (huile de neem pure Neemix) avec deux insecticides de synthèse (Fénitrothion et Deltaméthrine) a donné qu'aux doubles : impact sur l'environnement et coût des traitements, l'utilisation des produits naturels à base de neem apparaît plus rentable contre les larves du moustique *Culex quinquefasciatus*.

Mots clés
Azadirachta indica, Culex quinquefasciatus, Fénitrothion, Deltaméthrine, effets larvicides

Abstract
With the drawbacks of most synthetic chemical insecticides, Scientists are in searching other insecticidal formulations as neem (*Azadirachta indica*) products. For that, various chemical industries have been formulated Azadirachtin-based products. Laboratory bioassays showed that Neem oil and Neemix formulated by a senegalese industry (SENCHIM) were very toxic against *Culex quinquefasciatus* larvae. Under laboratory conditions, the LD100 (lethal Dose 100) of neem oil was 9% in 24h, and 0.028% for Neemix. However, our study revealed in an unexpected way that solvent used (Solvesso 100) for Neemix formulation was very toxic for mosquito larvae. In addition, the comparison between neem products (neem oil and Neemix) and two synthetic insecticides (Fenitrothion and Deltamethrin) revealed by considering the environment impact and treatments cost, it is desirable to investigate the neem products usefulness against mosquito larvae *Culex quinquefasciatus*.

Keywords :
Azadirachta indica, Culex quinquéfasciatus, Fenitrothion, Deltamethrin, Larvicidal effects

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INTRODUCTION

En dehors de leur nuisance, les moustiques causent aussi des maladies vectorielles. Les principaux genres de moustique sont : *Anopheles*, *Aedes*, *Culex* et *Mansonia*. Dans les pays en développement, les maladies vectorielles les plus fréquentes sont le paludisme (malaria) et la fièvre jaune. Le moustique *Culex quinquefasciatus* est le principal vecteur de la Filariose de Bancroft et de maladies virales comme les encéphalites.

Dans le cadre de la lutte contre ces maladies vectorielles, des quantités très importantes de larvicides sous forme de produits chimiques de synthèse sont déversées dans le milieu aquatique. Cependant, en dehors de leur toxicité, certains produits chimiques utilisés dans cette lutte sont devenus moins efficaces du fait de la résistance développée par certains moustiques (OMS [1]; Chandre et **al.** [2]; Rodriguez *et* **al.** [3], Konan et **al.**[4]). A cela, s'ajoute la résistance du parasite *Plasmodium falciparum* (responsable du paludisme) à la chloroquine (Nuwala [5]).

Les Scientifiques tentent alors de trouver d'autres produits accessibles, moins toxiques (produits naturels surtout) pour mener cette lutte. D'importants résultats ont été trouvés dans le cadre de l'utilisation des produits naturels contre les insectes. Le neem est généralement utilisé dans la lutte contre les insectes. L'effet antifertilisant de l'huile de neem a été démontré sur le rat mâle (Upadhyay [6]). Les travaux de Koua [7] ont montré l'effet larvicide d'une plante *(Persea americana)* sur des larves du moustique *Anopheles gambiae*. Pushpalata et Muthukrishnan [8] ont montré l'effet insecticide d'autres plantes tropicales sur des larves de moustique. En plus de l'activité larvicide, le neem a une action d'oviposition négative sur les moustiques (Tianyun et Mir [9]). Sa principale matière active est l'Azadirachtine mais d'autres substances (triterpenoïdes) ont montré leur activité insecticide (Bina [10]). Le neem agit par ses effets antiappétants, répulsifs (Ravindra et **al.** [11]) et d'inhibition de la croissance des insectes. L'effet larvicide des différentes pièces du neem (feuilles, graines et tige) sur des larves d'anophèles a été démontré par Aliero [12]. Des extraits aqueux de neem ont montré des effets néfastes sur la fertilité, la fécondité et le développement post-embryonnaire de certains diptères (Shivendra [13]). Par ailleurs, la comparaison des produits naturels avec les produits chimiques de synthèse permettrait de mieux valoriser ces insecticides naturels, même si l'utilisation de produits naturels n'est sans risques. Une étude comparative d'insecticides naturels et chimiques de synthèse dirigée par Mittal et **al.** [14] a montré que l'utilisation de la Deltaméthrine a été plus toxique sur les poissons que l'utilisation des biocides (Spheris et bactoculicide). Le Fénitrothion a une capacité de perturber la différentiation sexuelle androgène-dépendante chez le rat (Turner et **al.** [15]) et a un effet toxique chez des bivalves marins (Pena-Lopes et **al.** [16]). A une certaine dose, il a une toxicité aiguë sur *Corydoras paleatus* (Sarikaya et **al.** [17]).

Dans nos travaux, nous avons fait des investigations sur l'effet larvicide de deux produits de neem (Neemix et huile de neem pure), ensuite nous les avons comparés à deux insecticides chimiques de synthèse (Deltaméthrine et Fénitrothion).

MATERIEL ET METHODES

Les produits utilisés

Les produits (huile de neem pure 0,3g/l, Neemix @ 1%, Deltaméthrine 25% et Fénitrothion 4%) sont fournis par une industrie chimique Sénégalaise : la SENCHIM. La Deltaméthrine est une formulation CE 25% (Concentrée Emulsionnable 25%). A partir du Fénitrothion 4% (en poudre), nous avons fait une bouillie 0,08% (p/v) en mélangeant 2g de produit dans 10ml d'eau.

Matériel biologique

Des oeufs de *Culex quinquefasciatus* sous forme de barquettes sont récoltés au niveau des bacs du département de Biologie Animale. Ces œufs sont mis à l'éclosion dans des bocaux remplis d'eau du robinet. Après l'éclosion, les larves de premier stade sont nourries avec de la poudre de pain en prenant le soin de changer l'eau de culture deux fois par semaine pour empêcher un développement éventuel des champignons qui pourraient les tuer. Après chaque mue, nous avons isolé les larves de même génération dans un autre milieu de culture. Nous avons obtenu ainsi successivement les larves de stades I, II, III, et IV séparées par des mues.

Traitement avec les huiles

Nous avons utilisé 11 bocaux de 10 cm de côté et 7 cm de hauteur.

Avec les produits de neem, nous avons traité dans les 10 premiers bocaux les larves avec les produits en prenant des doses correspondant à des concentrations en matière active pour chaque produit. Nous avons utilisé des pas allant de 0,1ml à 0,19ml pour le Neemix et de 5 à 50 ml pour l'huile pure. Ces valeurs sont choisies dans le but de chercher les doses létales 100 (DL100). Après chaque traitement, les constats de mortalité sont faits toutes les 24 h et le nombre cumulé de larves mortes est consigné dans un tableau. Nous avons effectué deux séries de tests, les valeurs représentent la moyenne des deux séries.

Pour calculer le pourcentage de mortalité (mortalité corrigée) des larves, nous avons appliqué la formule d'Abbott (Abbott, [18]).

$$\%\ m = \frac{NLm - NLT}{NLtotal - NLmT} \times 100$$

%m = pourcentage de mortalité
NLm = nombre de larves mortes
NLtotal = nombre de larves total
NLmT = nombre de larves mortes dans le témoin

Traitement de comparaison

Pour la comparaison des différents produits, nous avons fixé la quantité de matière active (ma) à une dose légèrement supérieure à la CL50 de la Deltaméthrine (6mg/l). Avec cette dose, nous avions eu une mortalité de 59,4% en 24 h au laboratoire. Nous avons fait 4 séries de tests. Pour chaque série, nous avons utilisé 6 bocaux avec 25 larves et 500ml d'eau. Les quatre bocaux ont servis à tester les produits, les deux autres étant les témoins (solvant et eau). Nous avons fait un constat de mortalité 24 h après pour chaque bocal. Les résultats obtenus sont traités avec le test d'ANOVA et présentés sous forme de figure.

RESULTATS :

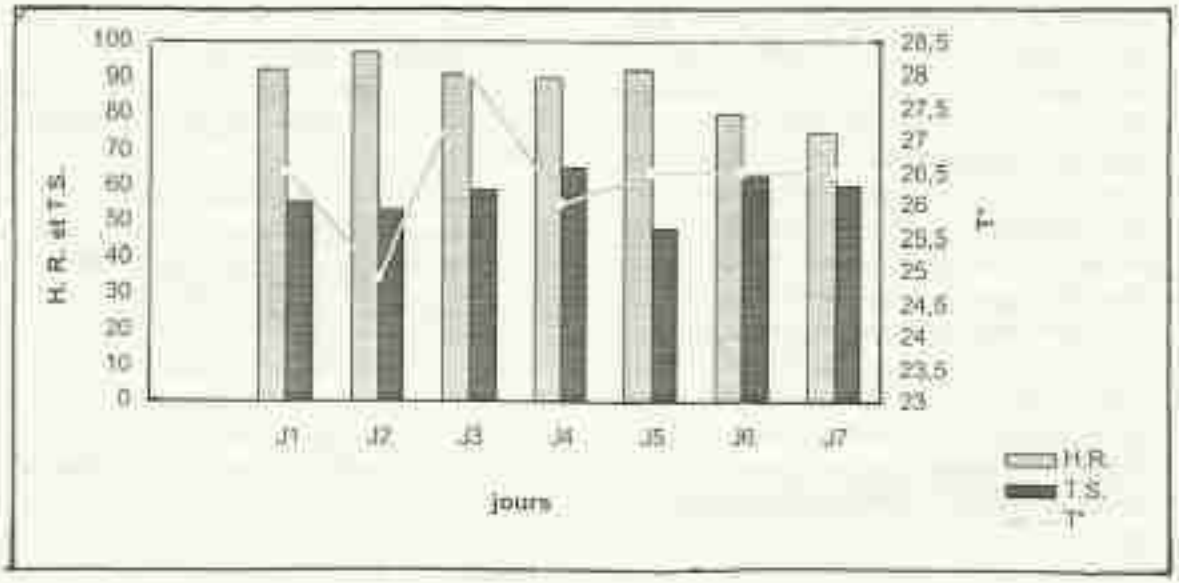

Figure 1 : Conditions de la première série de traitement

Moyenne Température = 26 °C, Ecart moyen = 0,5
Moyenne Humidité relative = 88%, Ecart moyen = 6
Moyenne Taux de saturation = 57 %, Ecart moyen = 4

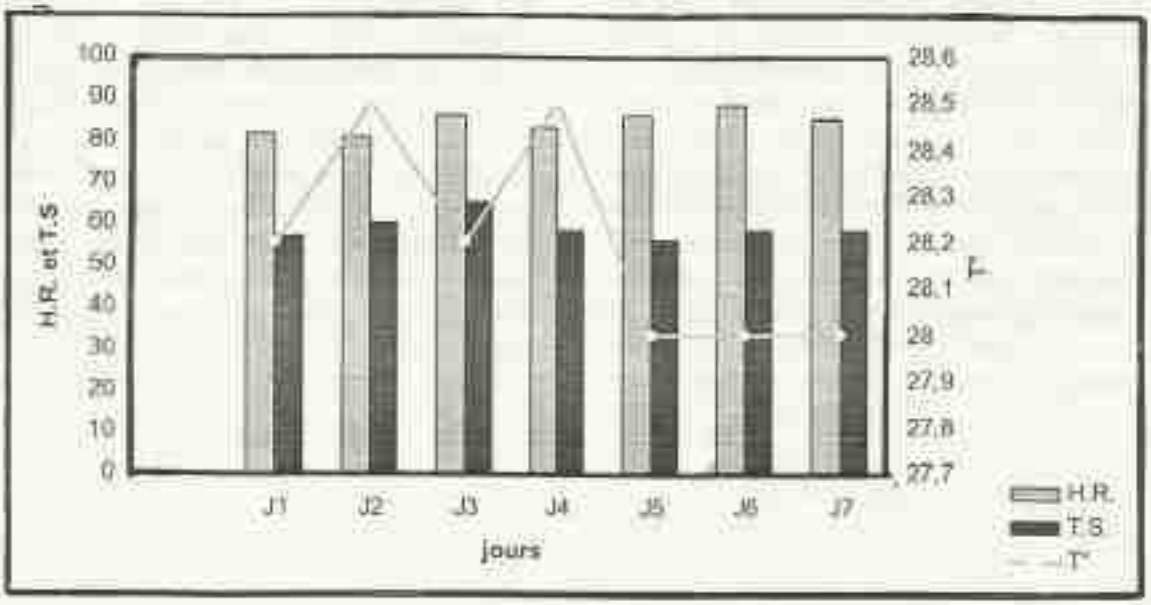

Figure 2 : Conditions de la deuxième série de traitement

Moyenne température = 28°C, Ecart moyen = 0,1
Moyenne Humidité relative = 84%, Ecart moyen = 2
Moyenne Taux de saturation = 58 %, Ecart moyen = 3
H.R. = Humidité Relative, T.S. = Taux de Saturation, T° = Température

Tableau I : mortalité des larves de *Culex quinquefasciatus* traitées avec l'huile de neem pure 0,3g/l

Jours	Pourcentages en Huile de neem pure (%)										Bocal témoin
	1	2	3	4	5	6	7	8	9	10	
J1	72,35	58,15	73,8	86,5	80,75	88,75	95,40	98,45	100	100	1
J2	90,95	85	90	91	98,5	100	100	100	-	-	1
J3	95,80	86,95	95,3	95,8	100	-	-	-	-	-	3
J4	97,27	93,95	97,25	96,15	-	-	-	-	-	-	5,5
J5	98,85	94,25	97,25	97,7	-	-	-	-	-	-	7,5
J6	98,85	94,8	97,25	98,25	-	-	-	-	-	-	7,5
J7	98,85	95,85	98,2	98,25	-	-	-	-	-	-	8,5

Tableau II: mortalité des larves de *Culex quinquefasciatus* traitées avec le Neemix 1%

Jours	Pourcentages en Neemix (%)										Bocal témoin
	0,020	0,022	0,024	0,026	0,028	0,03	0,032	0,034	0,036	0,038	
J1	56,7	69,3	85,5	91,4	100	100	100	100	100	100	40
J2	78	80,7	92,3	96,7	-	-	-	-	-	-	43
J3	81,3	83,3	92,3	96,7	-	-	-	-	-	-	45,5
J4	87,1	89,2	92,3	96,9	-	-	-	-	-	-	48
J5	88,9	92,6	93,3	97	-	-	-	-	-	-	49
J6	92,5	94,7	96	98,4	-	-	-	-	-	-	49,5
J7	94,5	97,6	96	-	-	-	-	-	-	-	51

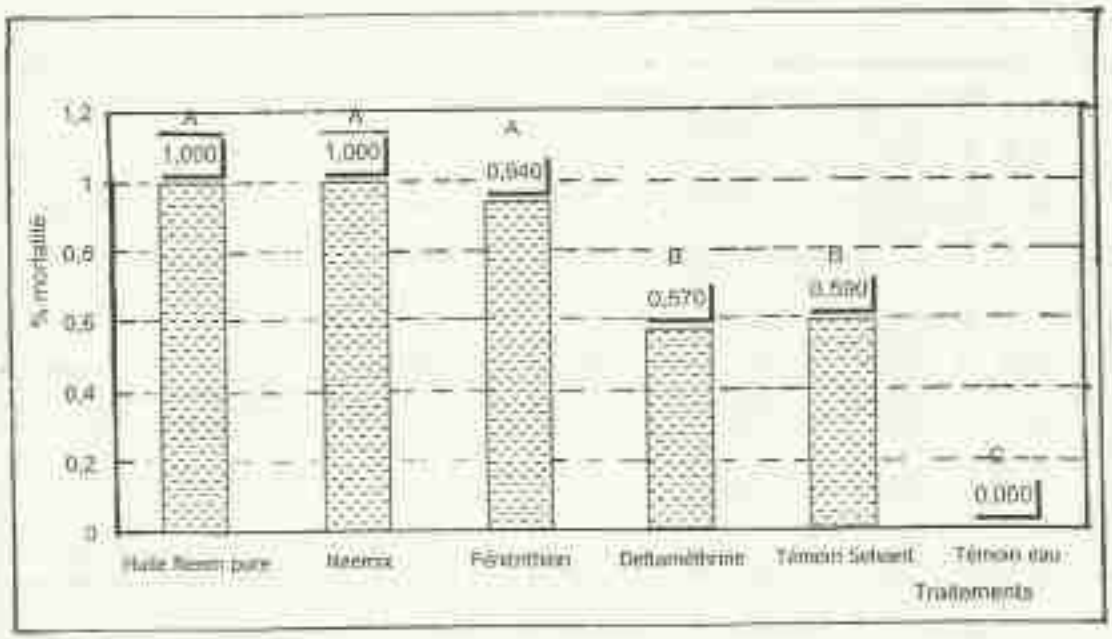

Figure 3 : Classement par groupe des produits naturels et chimiques de synthèse selon leur efficacité sur les larves

(Conditions de traitement : Température = 28°c, Humidité Relative = 88%, Taux de Saturation = 56%)

Groupe A : Huile de neem pure, Neemix, Fénitrothion
Groupe B : Deltaméthrine, Témoin solvant,
Groupe C : Témoin eau

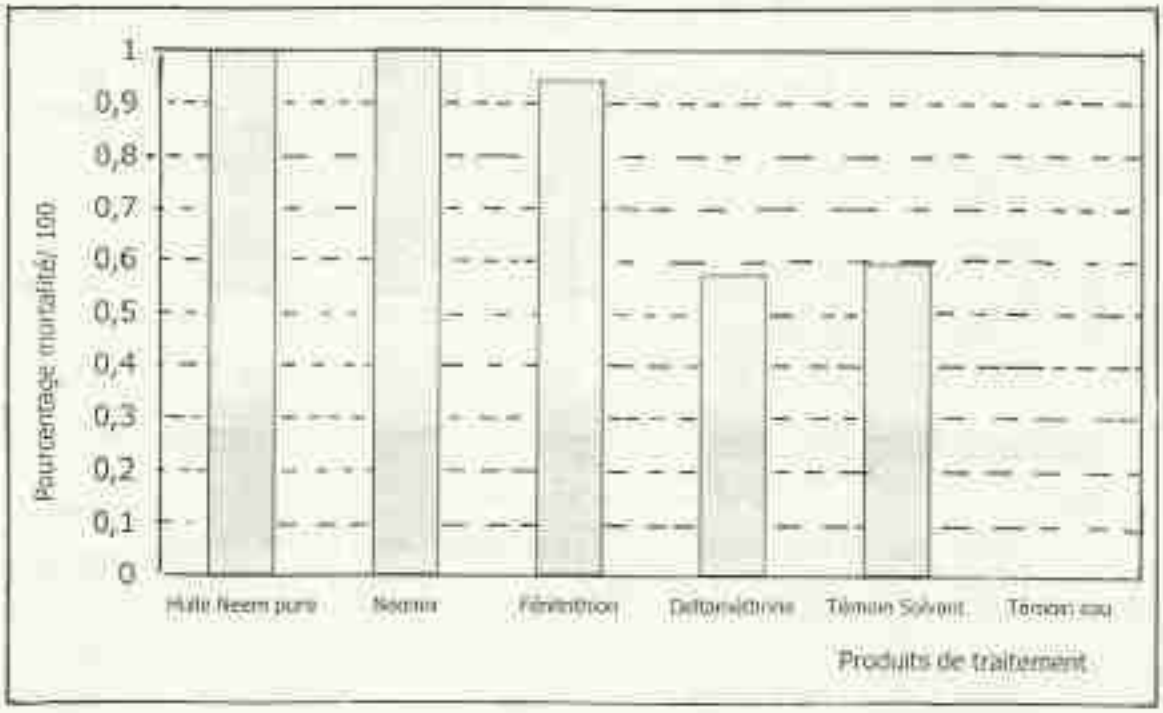

Figure 4 : Courbe des pourcentages de mortalité suivant les produits

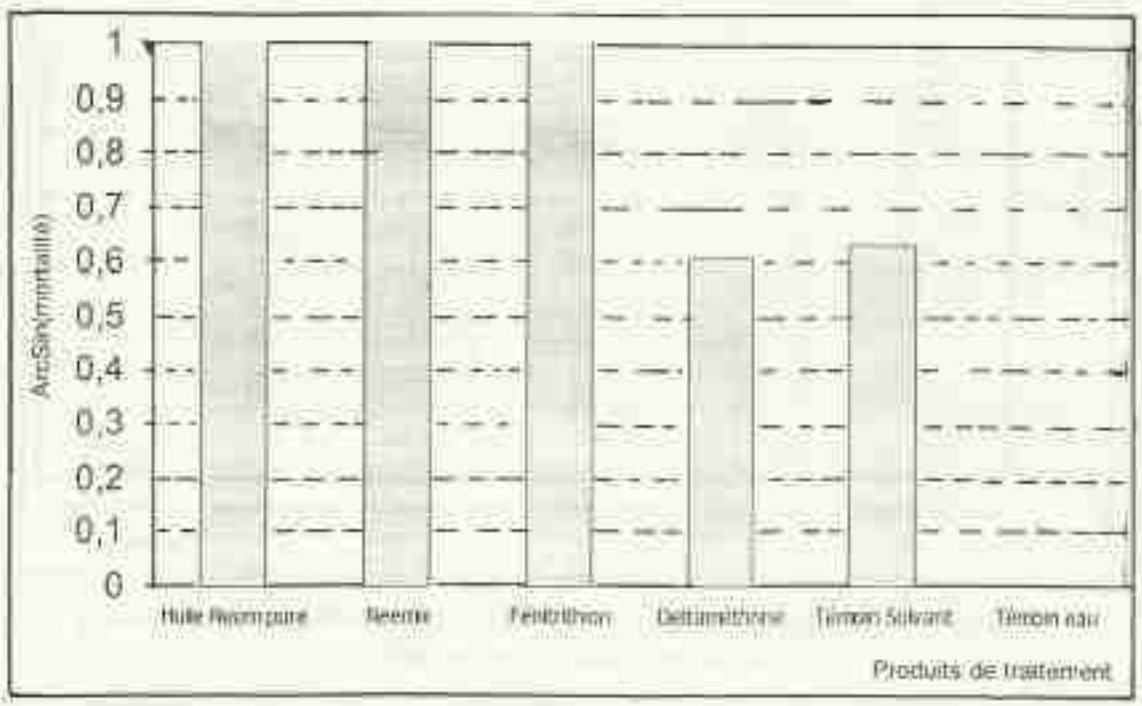

Figure 5 : Arc sinus des mortalités suivant les produits

Les résultats donnés par le traitement des larves avec les produits de neem dans les conditions du laboratoire (figures 1 et 2) montrent que la Dose létale100 (DL100) de l'huile de neem pure en 24 h est de 9% soit 2,47 mg/l (tableau I). Avec le Neemix, cette DL100 se situe à 0.028% soit une concentration de 2,8 mg/l (tableau II). Le taux de mortalité des larves 7 jours après traitement pour les deux produits montre des valeurs entre 94 et 98% (tableaux I et II). Cependant, la mortalité des larves au niveau des deux témoins (solvant et eau) indique des valeurs allant de 1 à 8,5 % pour le témoin eau et de 40 à 51% pour le témoin solvant.

Après application de l'huile de neem pure, les larves se regroupaient dans une zone où la couche huileuse était moins importante. La mort des larves s'est manifestée d'abord par une diminution progressive des mouvements et enfin une immobilisation complète au fond des bocaux. Au moment où les larves de stade avancé dans le bocal témoin (eau) passaient rapidement en nymphes puis en adultes, certaines traitées avec les huiles n'ont subies aucune mue et celles qui sont passées en nymphes n'ont pas pu s'échapper.

Avec le traitement de comparaison, le test d'ANOVA nous a permis de faire un classement des groupes non significativement différents avec un intervalle de confiance à 95% (fig. 3). Les résultats montrent que le solvant (Solvesso 100) est aussi toxique que la Deltaméthrine avec respectivement des pourcentages de mortalité 57% et 59%. Cependant, les mortalités pour l'Huile de neem pure, le Neemix et le Fénitrothion sont respectivement 100%, 100% et 94%. Les figures 4 et 5 montrent l'ordre de l'efficacité des produits de comparaison.

DISCUSSION :

L'application de l'huile de neem émulsionnable à 5% sur des larves de stades III et IV de *Culex quinquefasciatus* a entraîné des mortalités de 51,6 et 91,2% entre 1 et 14 jours (Batra et **al.** [19]). Les travaux de Aliero [12] ont montré que l'huile de neem à 20% a entraîné une mortalité à 100% des larves d'anophèle. Avec des larves de *Culex quinquefasciatus*, nous avons obtenu une mortalité de 100% en 24 h mais avec une dose de 9% soit 2,47 mg/l. Cependant, même à 1% soit 0,29 mg/l, nous avons obtenu 98,85% de mortalité des larves 5 jours après. Les travaux de Aliero [12] ont montré que l'huile des graines de neem était la plus mortelle parmi les diverses pièces de l'arbre neem. Nos résultats sur l'huile de neem pure ont montré que l'huile tue non seulement par ses composés chimiques (matières actives), mais aussi par une insuffisance d'oxygène. En effet, l'huile pure forme une couche à la surface de l'eau et empêche aux larves de respirer. Les travaux de Scott [20], ont montré l'effet insecticide d'une formulation du neem (Margosan-O®) sur des larves de *Culex sp.* en mixant 9,4 ml et 28,2 ml de ce produit avec 1 litre d'eau soit 10 et 30 mg/l. L'application de ce produit a entraîné en 13 h une mortalité de 100% des larves de *Culex* sp. avec la dose de 30 mg/l. Nos résultats avec le Neemix (R) ont donné le même taux de mortalité des larves de *Culex quinquefasciatus* mais en 24 h avec une dose de 0,028% soit 2,8 mg/l en Azadirachtine. La forte toxicité du Neemix (R) utilisé laisse croire que cette formulation est très efficace contre les larves de *C. quinquefasciatus*. Mais en fait, c'est le solvant utilisé (solvesso 100) qui est à l'origine de cette forte toxicité. L'action du solvant est révélée par la forte mortalité des larves dans le bocal témoin (40 à 51%) alors que le volume de solvant est faible (0,19ml du produit dans 500 ml d'eau). Ces résultats laissent supposer que certains insecticides chimiques formulés avec le Solvesso 100 sont efficaces du fait de l'effet toxique de ce solvant. Ce qui ne serait pas le cas avec l'éthanol déjà utilisé pour l'extraction de produit du neem (Carolina et **al.** [21]). Cependant, avec une dose faible

en Neemix® (0,02%) soit 0,2 mg/l d'azadirachtine, nous avons obtenu 7 jours après une mortalité larvaire de 94,5 %. A ce niveau, l'effet de la matière active a joué son rôle toxique en provoquant une mortalité au cours du temps (7 jours). Cela s'explique par le fait que la teneur de ce produit en matière active (m.a.) est plus élevée (10 g/l) que l'huile de neem pure (0,63g/l). Néanmoins, la formulation d'autres produits à partir de cette huile pure peut améliorer son efficacité. Les fortes mortalités dues aux produits de neem sur les larves de *Culex quinquefasciatus* par rapport à la Deltaméthrine s'expliquent par le fait que l'huile de neem pure tue déjà à 100% pour une dose de 9% en 24 h soit 2,22 mg/l. Le Neemix tue aussi à 100% pour une dose très faible 0,028% soit 0,28 mg/l. Etant donné que l'huile de neem pure tue par asphyxie, la quantité de ce produit joue plus que sa teneur en matière active. Cependant, il faut noter que l'utilisation du Fénitrothion à 1mg/l a un effet très toxique sur d'autres organismes non ciblés (Ferrando et **al.** [22]). Les travaux de Wang [23] ont montré que la CL50 (Concentration Létale 50) de la Deltaméthrine sur les larves du moustique *Anopheles sinensis* était de 0,0209 ppm. Alors qu'avec les larves de *Culex quinquefasciatus*, nous avons obtenu 57% de mortalité pour une concentration de 6 mg/l soit 300 fois la CL50 obtenue par Wang [23]. Ce qui fait penser à une possibilité de résistance ou à une différence de sensibilité entre ces deux espèces vis à vis de la Deltaméthrine. Les travaux de Kreutzweiser et **al.** [24] ont montré qu'une formulation à base d'azadirachtine (Neemix® 4.5) n'a un effet toxique sur le zooplancton qu'avec des doses supérieures à 10 g/l. Ce qui est largement supérieure aux doses de produits de neem que nous avons choisies. Sur le plan environnemental, les produits chimiques de synthèse sont plus rémanents que les produits à base de neem. Les travaux de Szeto et Wan [25] ont montré que la demi-durée de vie de l'Azadirachtine est comprise entre 44 et 282 h pour des températures comprises entre 25 et 35°c. Ce qui montre l'effet des produits de neem au cours du temps (7 jours) dans les conditions du laboratoire (entre 24 et 28°c).

Par ailleurs, le prix commercial de la Deltaméthrine Decis EC 25 SC est aux environs de 7€ le litre, la Deltaméthrine K'othrine 24 € le litre, alors que certains insecticides à base de neem ont un prix compris entre 11,9 et 56,5€ le litre, selon la formulation (d'après www.city plantes.com). Du fait de la baisse d'efficacité de certains insecticides chimiques, ces derniers sont utilisés en grande quantité pour avoir des résultats satisfaisants dans la lutte chimique. Cependant, le prix à payer pour l'utilisation de certains produits chimiques de synthèse toxiques est plus important que le prix d'achat des produits de neem. Sur le plan socio-économique et environnemental, il serait plus rentable d'utiliser ces produits naturels dans la lutte chimique, d'où un apport important dans le domaine de la lutte intégrée.

Remerciements :

Les auteurs remercient les autorités de la SENCHIM en particulier Docteur Abdoulaye DIEYE. Ils remercient également Docteur Saliou NDIAYE (ENSA de Thiès) pour son assistance dans le traitement statistique.

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Biology and Medicine

E-ISSN: 0974-8369

Research Article

Histopathological Effects of *Cymbopogon citratus* (Lemongrass) Essential Oil on Late Third Instar Larvae of *Aedes aegypti* L. (Diptera: Culicidae)

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ABSTRACT

The essential oil of *Cymbopogon citratus* is generally used against mosquitoes. The aim of this study was to evaluate the larvicidal and histological effects of *C. citratus* essential oil against *Aedes aegypti* larvae. Third larval stages were exposed at 0.2 to 1 µl/ml doses, and mortality was recorded at 4, 12, and 24 h. The histopathological study was assessed for the control and treated larvae at 4, 12, and 24 h using light and electronic microscopies. The results show that larval mortalities were 19.4±2.3%, 43.2±3.7%, 70.3±2.6%, 92±4.5%, and 100±0.0% for respectively 0.2, 0.4, 0.6, 0.8, and 1 µl/ml doses 24 h after treatment compared to the control (01.4±0.0%). Histological studies with light microscope revealed disruption of the cellular structures of midgut, damaged of apical surface toward the gut lumen, disorganization of muscle and fat body compared to the control. Electron microscopy revealed that the product acts by destroying intestinal microvilli, with cytoplasm being highly vacuolated. The sarcoplasmic reticulum, mitochondria, and genetic material were also disorganized. At the same time, the muscular tissue was progressively disorganized, and the actin and myosin filaments were transformed into vacuoles. Thus, as a biological product, preparation-derived extract of *Cymbopogon citratus* could be used as an effective botanical larvicide in mosquito control such as *Aedes aegypti*, *Anopheles*, and *Culex* species.

Keywords: *Cymbopogon citratus*; Biopesticide; *Aedes aegypti*; Histopathology

INTRODUCTION

Many mosquito (Diptera: Culicidae) species are vectors responsible for the transmission of infectious diseases of medical importance. These mosquito-borne diseases significantly contribute to disease burden, death, poverty, and social debility in many developing countries such as Senegal. *Aedes aegypti* is an epidemic vector of several diseases such as dengue fever, yellow fever, and Zika virus. Because of the mosquito resistance to some pesticide [1], plants extracts are commonly used for mosquito control [2-4]. Among them, *Cymbopogon citratus* oils were investigated as mosquito repellents [5-7] and as antimalarial product [8].

The essential oil hydrolates were investigated against mosquito larvae and are very promising [9].

Using biological products, citronella oil is one of the essential oils extracted from different species of *Cymbopogon* against insect. Without the repellent effect, *C. citratus* oil has larvicidal effect. *C. citratus* also causes significant growth inhibition and mortality in later developmental stages of *A. aegypti*. However, essential oils have repellent effect on adults and are very volatile, and that is why larvicidal effects should be more effective in mosquito control.

Some plant extracts were investigated against *Aedes aegypti* larvae [10]. Most larvicides from plant extract act after ingestion by histological alteration [11-16].

In this present study, cytotoxicity of *C. citratus* essential oil was evaluated against *A. aegypti* larvae, and histopathological effect was evaluated with light and electronic microscopy.

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Received: January 08, 2021, **Accepted:** February 06, 2021, **Published:** February 26, 2021.

Citation: Seye F, Fall A, Toure M, Ndione RD, Ndiaye M (2021) Histopathological Effects of Cymbopogon citratus (Lemongrass) Essential Oil on Late Third Instar Larvae of Aedes aegypti L. (Diptera: Culicidae). Biol Med (Aligarh) 13: 100287.

E-ISSN: 0974-8369

MATERIALS AND METHODS

Mosquito larvae

Mosquito larvae were collected from natural vats at the Animal Biology Department, Cheikh Anta Diop University. They were selected during first and second stage and cultivated until the third stage. They were reared at a temperature of 28±3°C and relative humidity of 75± 9%.

Cymbopogon oil

The cymbopogon oil was obtained from the Chemical Department, Faculty of Science and Technology, University Cheikh Anta Diop of Dakar. It was obtained by mechanical extraction with steam distillation of the dried leaves and solubilized in Tween 20.

Toxicity tests

Conventional toxicity tests were first conducted in aqueous suspensions according to the World Health Organization protocol [17] to assess lethal concentrations of *Cymbopogon citratus*. Twenty-five larvae were transferred to separated bottles containing 100ml of distilled water. Five concentrations of 0.2, 0.4, 0.6, 0.8 and 1 µl/ml were applied, and the control group was not treated. The bioassay was maintained at a temperature of 28±3°C and relative humidity of 75±9%. Larval mortality was recorded at 4, 12, and 24 h after treatment. We considered that larva were dead or moribund when they did not move. The mortalities were corrected using Abbott's formula [18]. Treatments were replicated four times at different time intervals.

Histopathology with light microscopy

The larvae were fixed in carnoy for 24h, dehydrated through a graded ethanol series of increasing concentrations (70%, 80%, 95%, and 100%), and cleared with xylene solution. They were embedded in a block using paraffin at the embedding station. The blocks were sectioned at 5-µm thickness using a microtome. They were stained with hematoxylin and eosin. The glass slides were examined with microscope, and pictures were captured by using Leica DM500 digital camera.

Histopathology with transmission electronic microscopy (TEM)

For the electron microscopy, treated and control larvae were cut behind the head and in front of the respiratory siphon to facilitate the permeation of chemical products as described in our previous works [16]. Body segments were fixed for 12h in a 2.5% glutaraldehyde solution buffered with 0.1 M sodium cacodylate at pH7.3. They were then rinsed and stored for a few days in 0.2 M cacodylate buffer, before postfixation in 1% osmium tetroxide (1h) and three rinses (3 to 10 min) in distilled water. Dehydration was performed through a graded ethanol series of increasing concentrations: 30%, 50%, 70%, 90% (1 to 10 min), and 100% (3 to 20min).

Body segments were embedded in epoxy resin (SPI-PON 812, SPI-CHEM, SPI supplies, Leuven, Belgium) with propylene oxide as intermediate solvent for impregnation (2 to 30min in pure solvent, 2 h 30 min in solvent/resin mixture, and overnight in pure resin). Embedding was performed in flat silicone molds to facilitate sample orientation for sectioning and then placed in a stove (60°C, 72 h) to allow polymerization. Semithin (1–2mm) and ultrathin (60–80 nm) sections were performed between the second and fourth abdominal segments using a diamond knife on a Reichert-Jung Ultracut E (Reichert-Jung, Vienna, Austria) ultramicrotome. Semithin sections were stained with 1% toluidine blue (pH9) before observation for general histology and orientation of further ultrathin sections, using an Olympus Provis Ax70 (Olympus, Tokyo, Japan) light microscope equipped with an Olympus DP50-CU (Olympus) digital camera. Ultrathin sections were contrasted with uranyl acetate and lead citrate according to the conventional method and then observed in a Jeol JEM 100-SX (JEOL, Tokyo, Japan) transmission electron microscope under 80kV accelerating voltage.

RESULTS

Toxicity of *Cymbopogon citratus* oil against *Aedes aegypti* larvae

The toxicity effect of various doses of *Cymbopogon citratus* oil is shown in Table 1. Larval mortalities increase with time of exposure and doses. At 24 h, the mortalities were 19.4±0.23% (0.20 µl/ml), 43.2±3.7% (0.40 µl/ml), 70.3±2.6% (0.60 µl/ml), 92±4.5% (0.80 µl/ml), and 100±0.0% (1.0 µl/ml) compared to the control (01.4±0.0%).

Histopathology of *Aedes aegypti* larvae treated with essential oil of *Cymbopogon citratus*

Light microscopy

With the control group (Figure 1A and 1B), we show the normal appearance of larval tissue. The epithelial cells are intact with the nucleus and the brush border surrounding the alimentary canal in the intestinal lumen. It also appears as a normal muscle and the fat body mass underneath the epidermis.

Regarding the same region of larvae treated with essential oil of *Cymbopogon citratus*, longitudinal section revealed an early

Table 1: Percentage mortality of *A. aegypti* larvae exposed to various doses of *Cymbopogon citratus* essential oil.

Time (hours)	Doses (µl/ml)					Control
	0.20	0.40	0.60	0.80	1.0	
4	06.5 ± 1.1	14.1 ± 0.2	21.4 ± 2.3	65.2 ± 4.1	87.8 ± 3.3	00 ± 0.0
12	11.2 ± 0.5	31.3 ± 3.4	56.7 ± 1.2	86.3 ± 6.3	95.5 ± 4.6	0.2 ± 0.0
24	19.4 ± 2.3	43.2 ± 3.7	70.3 ± 2.6	92 ± 4.5	100 ± 0.0	01.4 ± 0.0

Values are mean-corrected percent mortality of four replicates with standard error (SE).

E-ISSN: 0974-8369

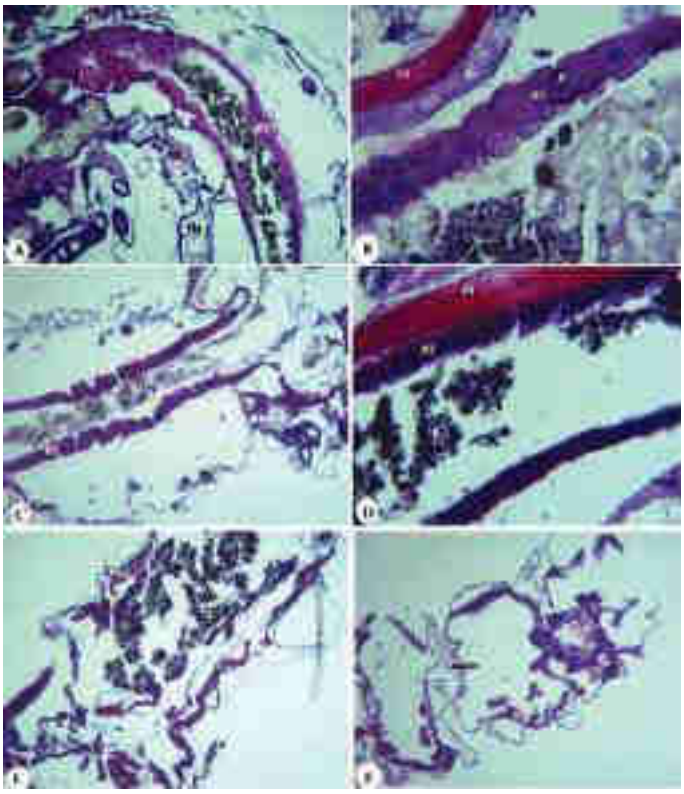

Figure 1: Histology of the whole body of *Aedes aegypti* control larva X100 (A), X400 (B) and treated larvae at 4 h X100 (C), X400 (D), 12 h X100 (E), and 24 h X100 (F) in longitudinal section. Abbreviation: epithelial cells (ec), intestinal lumen (lu), muscle (m), fat body (fb).

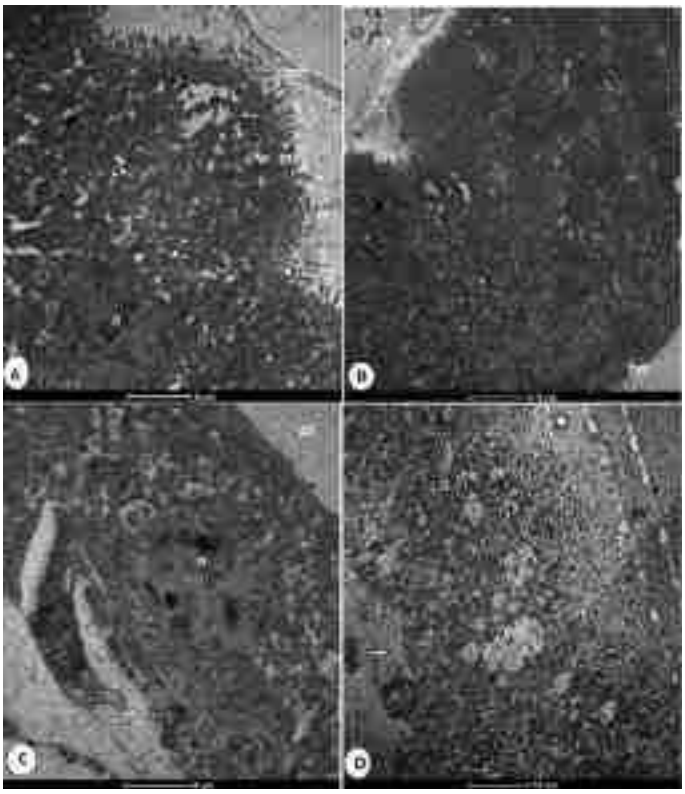

Figure 2: TEM microphotographs of digestive epithelial cells in control (A) and treated larvae at 4 h (B), 12 h (C), and 24 h (D). Abbreviation: nucleus (n), peritrophic membrane (pm), basal lamina (bm), peritrophic space (ps), vacuolization (v).

degradation of tissue as intestinal gut and fat body mass 4 h after treatment (Figure 1C and 1D). The disorganization is more pronounced at 12h (Figure 1E) with broken intestinal layer and completely disrupted muscle. Twenty-four hours after treatment (Figure 1F), larval body is disintegrated, digestive tract is completely disappeared, and larval tissues are completely disorganized.

Transmission electronic microscopy

The digestive tract

In control larvae, microvilli of epithelial cell with prominent nucleus are well organized near the peritrophic membrane (Figure 2A). Four hours after treatment (Figure 2B), the microvilli appears less abundant at the epithelial cells than control, but the basal lamina is intact. At 12 h (Figure 2C), disorganization appears at the basal lamina (arrow), and microvilli are completely destroyed at the peritrophic space with a beginning of vacuolization in the cytoplasm. The vacuolization is more pronounced at 24 h (Figure 2D).

The muscle

In the control group (Figure 3A), the myofibrils are well organized, and nucleus is present at the periphery. After larvae are treated, the myofilaments actin and myosin surrounded by sarcoplasmic reticulum disappear (arrow) between 4 h (Figure 3B) and 24h (Figure 3D). Progressively, vacuolization appears in the sarcoplasmic area.

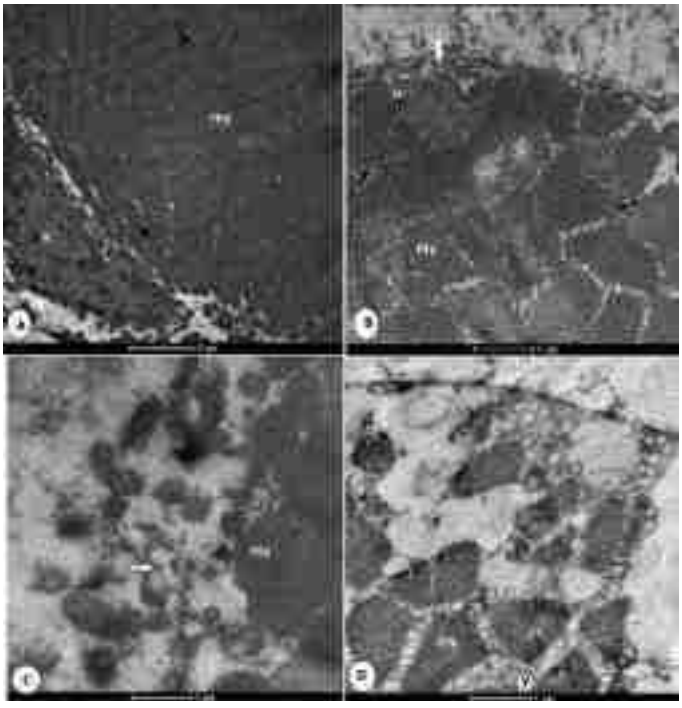

Figure 3: TEM microphotographs of cross-sectioned striated muscle in control larvae (A) and treated larvae at 4 h (B), 12 h (C), and 24 h (D). Abbreviation: nucleus (n), myofilaments (my), sarcoplasmic reticulum (sr), vacuolization (v).

E-ISSN: 0974-8369

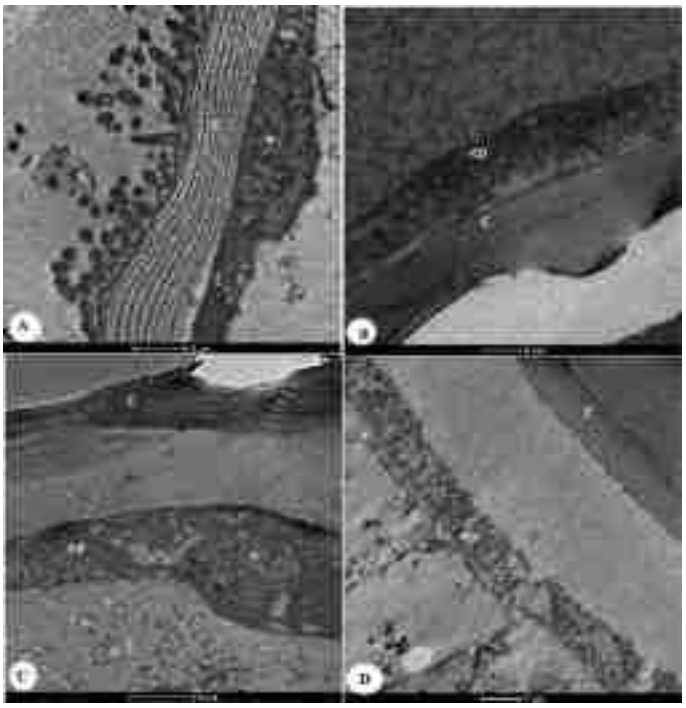

Figure 4: Epidermal cuticle of control (A) and treated larvae at 4 h (B), 12 h (C), and 24 h (D). Abbreviation: cuticle (c), epidermal cuticle (ep), nucleus (n).

The epidermal cuticle

Compared to the control group (Figure 4A), epidermis was progressively detached to the cuticle between 4 h (Figure 4B) and 24 h (Figure 4D) after treatment. Epidermal cells were destroyed with nucleus and epithelial cell disorganization.

DISCUSSION

In this present study, larvicidal activity of *Cymbopogon citratus* oil against *Aedes aegypti* was recorded on the third larval stages through the exposure period of 24h. With the exposure period, larval mortality increases from 6 to 80% at 4 h and 19 to 100% at 24 h for doses between 0.2 and 1 µl/ml. This toxicity confirmed the larvicidal effect of *C. citratus* oil against *A. aegypti* larvae as reported in the literature [9, 19, 20].

With 1 µl/ml, the mortalities recorded on *A. aegypti* were 87.8±3.3% at 4h and 100% at 24h, which were almost similar to results of others authors [20] who obtained 87.7±7.0% at 4 h and 96.6±5.7% at 24h after exposure.

Several studies have focused on natural products for controlling mosquitoes as larvicides [11, 12, 21-25]. *Cymbopogon citratus* oils have same effects against mosquito larvae and could be used as biopesticides. However, the effectiveness of *C. citratus* oil is different from that of the formulation with others oils [26]. In addition to repellent effect of *C. citratus* against mosquito bites reported by many authors [26, 27], the adulticide activity against *Aedes aegypti* has also been reported [6]. Depending on the mosquito stage, it is necessary to evaluate the choice between adult and larval control using essential oil. In fact, the repellent action of essential oil degrade after application [26] or the larvicidal action is an eliminatory processes of mosquito development.

To evaluate the process of plant extracts against insects, the most characteristic effects act most of the time at the digestive tract after ingestion [28, 29].

For our diagnostic of treated larvae, the present study highlights how *C. citratus* acts on the larvae compared to nontreated larvae. Indeed, the toxicity of *C. citratus* oil was confirmed on the larval tissues, and histological results revealed many damages.

The microscopic study revealed that *C. citratus* oil acts by destroying larval tissue cells. The most of the damage was observed at intestinal microvilli, mitochondria, genetic material, and the fat body mass. These histopatholigical results are similar to our previous results with other natural products [16, 30]. Most of natural products such as toxins are very toxic and cause damages at the intestinal tracts during exposure [16, 29]. Vacuolization and changes in the brush border of the midgut digestive cells of the *A. aegypti* larvae indicate that these cells were dying as reported by other authors.

In the present study, we even observed an epidermal damage with aspect being detached from the cuticle during exposure. This is similar to other work using a chitin synthesis inhibitor against *Aedes aegypti* larvae [31], which assumes that plant extracts may have an inhibitory effect on larval molting phenomena.

In addition to intestinal cell damages, larval muscle has been rigorously destroyed during 24-h exposure. The TEM revealed a disorganization of actin and myosin microfilaments, accompanied by a disappearance of sarcoplasmic reticulum. This is similar to that reported on *Culex quinquefasciatus* larvae treated with *Aspergillus clavatus* conidia [16]. Then, larvae were no more able to breathe air on the water surface and died due to suffocation.

CONCLUSION

The essential oil of *Cymbopogon citratus* was shown to be as potent as larvicidal activity against *A. aegypti* and caused 19.4%, 43.2%, 70.3%, 92%, and 100% mortality for respectively 0.2, 0.4, 0.6, 0.8, and 1 µl/ml doses in 24h. The essential oils act by destroying intestinal cells microvilli, muscle as well as the fat body mass. These results are very promising to use *C. citratus* oil for mosquito-borne disease control.

ACKNOWLEDGMENTS

We thank Dr. Thomas Bawin, University of Liege, and Pr. Philipe Compere, Department of Biology, Ecology and Evolution, Faculty of Sciences, University of Liege, for the technical assistance in the ultrastructural study. We also thank Pr. Frederic Francis, Department of Functional and Evolutionary Entomology, Gembloux Agro-Bio Tech, University of Liege.

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