USING MICRO-INJECTION TECHNIQUE TO ASSESS FUNGAL TOXICITY IN MOSQUITO CONTROL

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INTRODUCTION

Mosquitoes (Diptera: Culicidae) are zoonotic vectors responsible for numerous infectious diseases of medical and veterinary importance such as filariasis, malaria and many arboviruses (Goddard, 2008; Mullen and Durden, 2009). Integrated pest management is now promoted and entomopathogenic microorganisms are increasingly studied in a biological control context regarding their ability to infect and kill their host with more or less selectivity (Becker et al., 2010).

As part of a selection process, topical application of insecticidal compounds allows directly exposing them on insect tissues and measuring their toxicity while ignoring many factors (Yu, 2008; Ravindran et al., 2010). However, this technique remains difficult to apply on mosquito larvae considering their aquatic lifestyle. In this context, micro-injection could be used for the direct deposition of toxic compounds in the larvae (i.e. in the hemocoele through the cuticle, in the digestive tract or the respiratory system by respective orifices). In the case of pathogenic microorganisms, this method would provide (1) a negative or positive response (a strain is or is not pathogenic), (2) a minimum toxic dose which would compare the virulence between several microbial strains in standardized conditions, and (3) an estimation of the potential sites of action. Micro-injection has already been used to highlight the pathogenicity of bacteria (Misch and Anderson, 1986; Misch et al., 1987) and viruses (Becnel and Pridgeon, 2011) to mosquito larvae.

Among entomopathogenic microorganisms, many species of fungi have been showed to be of great interest in mosquito control (Scholte et al., 2004). The potentialities of this study will be evaluated for the estimation of the toxicity of entomopathogenic fungi.

MATERIAL AND METHODS

Fungal strains

Aspergillus clavatus (Desmazieres) (Seye and Ndiaye, 2008; Seye et al., 2009) and Metarhizium anisopliae ((Metschnikoff) Sorokin) (Seye et al., 2012, 2013) used in this study were both isolated from Oedaleus senegalensis (Krauss) (Orthoptera: Acrididae). Metarhizium sp. was isolated from Agriotes lineatus (Linnaeus) larvae (Coleoptera: Elateridae). All strains were grown in Petri dishes on PDA agar.

Mosquito rearing

Culex quinquefasciatus (Say) adults (S-Lab) were reared in 50 x 50 x 50 (cm) cages (Bugdorm®) and fed with 10% sucrose. The female blood meal was done on artificial membranes (Hemotek® membrane feeding systems). Oviposition occurred in black troughs filled with distilled water. The egg pods were placed in plastic trays (30 x 15 x 10 (cm)) filled halfway with

distilled water. After hatching, larvae were fed with a mixture of flakes for tropical fish (Tetramin®) and powder of natural beer yeast (Biover®) (ratio 3:1). Rearing conditions were 25±2°C temperature, 75% relative humidity and 16L:8D photoperiod.

Micro-injection system

Capillaries exhibiting a 6mm length injection tip with an external diameter of 500µm have been designed from silica tubes (Model P-97 Flaming/Brown Micropipette Puller, Sutter Instrument Company; Program 2: Heat = 555, Pull = 60, Time = 250, Pressure = 500). For each treatment, a capillary is mounted on a pump (Nanoliter 2010, World Precision Instruments, Inc.) connected to a flow rate regulator (Micro4™, World Precision Instruments, Inc.). Culex quinquefasciatus larvae (3rd or 4th instars) were taken individually and injected with 500nl of injection solution (125nl/s).

Injection track

The distribution of spores (10^7 spores/ml) stained with methylene blue (1g/l) and injected into the body of larvae was observed according to the system described. A preliminary spore counting using a hemocytometer (Thoma®) allowed to state that the internal tip diameter had no effect on the spore doses injected.

Bioassays

The spores of entomopathogenic fungi (Aspergillus clavatus, Metarhizium anisopliae, Metarhizium sp.) have been suspended at a dose of 10^7 spores/ml in Ringer's solution (NaCl 47mM; KCl 183mM; Tris-HCl 10mM; pH 6.8) previously selected as harmless against mosquito larvae (data not showed). Culex quinquefasciatus larvae (3rd or 4th instars) were injected according to the system described. Each treatment was applied to groups of 10 larvae placed in a controlled room (25±2°C, RH 75%, photoperiod 16L:8D) for a total of 4 replicates for each fungal strain. Mortalities were recorded daily during 72h.

Statistical analysis

Corrected mortalities (Abbott, 1925) were converted as proportions (p = [0;1]) and normalized using an angular transformation (Dagnelie, 1970): $y = 2\arcsin(\sqrt{p})$. These data were then subjected to ANOVA-1 test. All analyses were performed using Statistica 9 software. Results were considered as statistically significant when the p-value of the analysis was less than 5% ($p \leq 0.05$).

RESULTS

Results showed that the methylene blue-colored solution spread immediately within the larval tissues despite low reflux, and spores were distributed over the whole body (Figure 1). Injection of Ringer's solution alone resulted in 7%, 10% and 17% of mortality after 24, 48 and 72h respectively. By contrast, the injection of Aspergillus clavatus, Metarhizium anisopliae and Metarhizium sp. spores induced corrected mortalities of 25%, 21% and 16% respectively after 24h. These mortality rates increased to 41%, 38% and 42% after 48h, then 62%, 53% and 57% after 72h, and differed statistically from control groups ($p < 0.001$) (Figure 2). However, no significant differences were observed between mortalities induced by the tested strains. Finally, post-mortem emergences of filaments from dead larvae were observed in the case of the three fungal strains.

Figure 1. Culex quinquefasciatus larvae injected of Aspergillus clavatus spores stained with methylene blue. (a) Capillary inserted into a larva. (b) Distribution of methylene blue in the body of a larva (arrows) just after injection (500nl). (c, d) Spores found in the thorax (arrow). (e, f, g) Spores found in the abdomen (arrows).

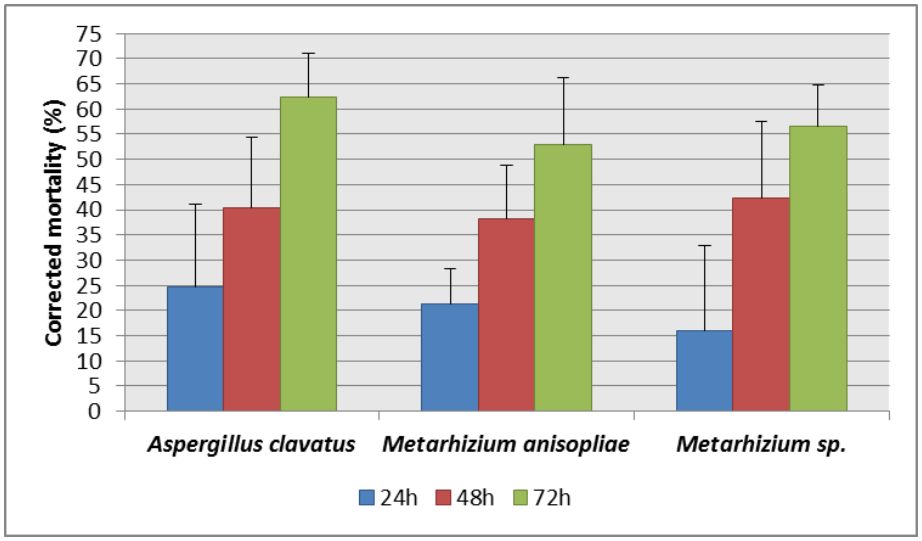

Figure 2. Corrected mortality (and standard deviation) induced by injection (500nl) of fungal strains at the dose of 10^7 spores/ml in Culex quinquefasciatus larvae (3rd and 4th instars).

DISCUSSION

In the described system, Ringer's solution was supposed to avoid osmotic stress since insect hemolymph is rich in amino acids, proteins, phosphate and trehalose (Wyatt and Kalf, 1957; Wyatt, 1961). However, newly drawn capillary tips were adapted to 6mm length and 500µm diameter in order to keep them sufficiently sharp to pierce the larval cuticle with the least possible damage, but thick enough to not (1) be plugged with spore solution and (2) impact the injected spore doses. As a result, control mortalities appeared unavoidable due to the trauma. Moreover, spores largely remained as aggregates when injected. Surfactants as Tween 80 would reduce surface tension in the capillary (thus allowing to decrease tip diameter) and improve the dissemination of the spores in the larval tissues.

Although corrected mortalities were statistically different from the control groups after 48h and 72h of spore injection, their pathogenicity against mosquito larvae cannot be established. Post-mortem emergences of filaments from dead larvae were observed in the case of the three fungal strains and confirming that the Ringer's solution had no effect on spore viability. But these observations did not exclude a saprophytic behaviour of the fungi. Moreover, no significant differences were observed between the tested strains. Injection of inactivated spores (or inert bodies of similar size) could help to prove the toxic effect for each fungal strain, and reject the hypothesis of a response due to the presence of foreign bodies.

The possibility of using this technique to determine specific lethal doses is related to the problem of the amount of spores contained in the injected volume since these remained as aggregates. One microliter of solution was sufficient to bring a larva in turgor, and 500nl caused only a slight swelling.

Other injection sites may eventually be tempted. Spore adhesion to the cuticle of larvae in conventional toxicity tests (i.e. in aqueous suspensions) is closely related to the type of formulation used (Lacey et al., 1988; Bukhari et al., 2011). The respiratory and digestive tracts are also significant infection sites (Lacey et al., 1988; Miranpuri and Khachatourians, 1991).

CONCLUSION

Micro-injection has already been used to highlight the pathogenicity of bacteria and viruses to mosquito larvae. In the present study, Culex quinquefasciatus larvae mortality could be related to the toxic effect of entomopathogenic spores (Aspergillus clavatus, Metarhizium anisopliae, Metarhizium sp.) after injection. Additional experiments (such as the injection of inactivated spores or inert bodies of similar size) are still needed to reject the hypothesis of a response due to the presence of foreign bodies.

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