Pathogenicity of *Metarhizium anisopliae* (Metch) Sorok and *Beauveria bassiana* (Bals) Vuill to adult *Phlebotomus duboscqi* (Neveu-Lemaire) in the laboratory

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ABSTRACT

Background & objectives: Biological control of sandflies using entomopathogenic fungi is a possible alternative to the expensive synthetic chemical control. It is potentially sustainable, less hazardous, and relatively inexpensive and merits further investigations. The objective of this study was to identify the most pathogenic fungal isolate(s) to sandflies in the laboratory.

Methods: Isolates of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* were screened for their pathogenicity against *Phlebotomus duboscqi*. Adult flies were contaminated using the technique described by Migiro *et al* (2010). Briefly, flies were exposed to 0.1 g of dry conidia evenly spread on a cotton velvet cloth covering the inner side of a cylindrical plastic tube (95 mm long × 48 mm diam). In all 25 sandflies were transferred into the cylindrical tube and allowed to walk on the velvet for one minute, after which they were transferred from the velvet into the cages in Perplex. Insects in the control treatments were exposed to fungus-free velvet cloth before being transferred into similar cages. The treatments were maintained at 25 ± 2°C, 60–70% RH and 12L: 12D photoperiod. The experiment was replicated 5 times. The most pathogenic isolates were selected for further studies.

Results: A total of 19 isolates were screened against adult sandflies in the laboratory. Mortality in the controls was approximately 16.8 ± 1.7 %. All the isolates were found to be pathogenic to *P. duboscqi*. Mortality ranged between 76.8 and 100% on all the fungal isolates tested. The lethal time taken to 50% (LT_{50}) and 90% (LT_{90}) mortality ranged from 3.0–7.8 days and from 5.3–16.2 days, respectively. The virulent isolates, causing mortalities of 97.5–100%, were selected for further studies.

Interpretation & conclusion: The high susceptibility of sandflies to entomopathogenic fungi suggests that fungi are potential alternatives to chemical control methods. We conclude that application of entomopathogenic fungi could result in acute mortalities of sandflies and reduction of parasite transmission and subsequently, reduction of leishmaniasis risk. This method of biological control has great potential as a new strategy for leishmaniasis control.

Key words   *Beauveria bassiana*; biological control; entomopathogenic fungi; *Metarhizium anisopliae; Phlebotomus duboscqi*

INTRODUCTION

Leishmaniasis is a disease caused by obligate intracellular protozoan of the genus *Leishmania* (Family: Trypanosomatidae)[1]. *Leishmania* infects humans, animals and sandflies[2]. The disease is transmitted by the bites of infected sandflies that belong to the *Phlebotomus* and *Lutzomyia* genera in the Old and New World respectively[3]. Though not as common as malaria, leishmaniasis continues to have a major impact on the world's population and is currently considered to be an emerging illness with high morbidity and mortality in the tropics and subtropics[4]. Leishmaniasis which is a neglected disease is found in 88 countries of the World and 350 million people are at a risk of getting infected[5]. There are 2 million new cases of leishmaniasis annually and 14 million people infected worldwide[5]. In the last decade, *Leishmania*-HIV co-infection has moved a notch higher as a major complication of leishmaniasis and ignited calls for the recognition of leishmaniasis as an AIDS defining illness[6]. In Africa, particularly Ethiopia and Sudan, it is estimated that 70% of adults with visceral leishmaniasis (VL) also have HIV infection[7].

Leishmaniasis is one of the most neglected tropical diseases, in terms of the few tools available for control and lack of clear criteria for methods of control[8]. The main control strategy of leishmaniasis is case finding and treatment plus, when feasible, vector control and in zoonotic foci, animal reservoir control[9]. Vector control with insecticides has been used in the endemic countries

with some degree of vector reduction[9–10]. In these resource restrained countries, vector control by spraying houses with insecticide is not sustainable due to logistical constraints and high cost[9]. In Kenya, most vector species such as the *P. duboscqi* are exophilic and exophagus, thus, negating common strategies such as indoor residual spraying (IRS) and insecticide-treated nets (ITNs)[11]. Furthermore, the use of Dichloro diethyl trichloroethane is prohibited in Kenya since it is one of the banned chemicals due to its potential for environmental damage. Alternative methods of vector control are therefore needed to control leishmaniasis in these economically poor regions. One such inexpensive and simple method is by use of entomopathogenic fungi.

In this study, we report the results of screening of 16 isolates of entomopathogenic fungi of *Metarhizium anisopliae* (Metschnikoff) Sorokin and three isolates of *Beauveria bassiana* (Balsamo) Vuillemin against adult *P. duboscqi* in order to select candidate isolates that will be developed as biological control agents for adult sandflies on account of their ability to kill them.

MATERIAL & METHODS

Sandflies

Adult *P. duboscqi* sandflies were obtained from colonies maintained in KEMRI, Nairobi, Kenya. Larvae and adults of *P. duboscqi* were reared according to the methods described earlier[12,13]. In all experiments, 3 to 5-day old adult sandflies were used.

Fungi

All the fungal isolates used in the study were obtained from the ICIPE's Germplasm Centre, except one isolate that was isolated from soil samples collected from Baringo district and given letter 'O' (Table 1). Isolates were cultured on Sabourand dextrose agar (SDA) in Petri dishes and incubated at room temperature (22–28ºC). Conidia were harvested by scraping the surface of three week old cultures. The viability of conidia was determined by spread plating 0.1 ml of conidial suspension (titrated to 3×10^6 conidia ml^{-1}) on SDA plates. Sterile microscope cover slips were placed on each plate. The plates were incubated at 24–29ºC and examined after 20 h. Percent germination was determined by counting approximately 100 spores for each plate at ×40 magnification. Each plate served as a replicate with four replications per isolate. Conidia were produced on long white rice substrate in plastic bottles (130 × 130 × 230 mm). The substrate was autoclaved for 1 h at 121ºC and inoculated with 3-day old culture of blastospores. The substrate was then incubated for 21 days under ambient conditions (20–26ºC, 40–70% RH) and then allowed to dry for 5 days at room temperature. Conidia

Table 1. Pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* isolates against sandflies

Fungal isolate	% Mortality ± S.E.	LT_{50} (days)	LT_{90} (days)	Slope	χ^2	Probability
Control	16.8 ± 1.7 g					
M. anisopliae						
A-ICIPE-41	95 ± 4.1[abcd]	4.5 (4.3–4.6)	10.8 (10.4–11.3)	0.11	1103.1	<0.0001
B-Gategi	89.2 ± 5.4[cde]	5.5 (5.3–5.7)	16.2 (15.3–17.3)	0.11	1080	<0.0001
C-MER-10	100 ± 0[a]	3.0 (3.0–3.1)	5.3 (5.2–5.5)	0.13	972.6	<0.0001
D-EMB-27	96.7 ± 2.1[abcd]	4.2 (4.1–4.3)	9.1 (8.8–9.4)	0.12	1194	<0.0001
E-ICIPE-18	92.5 ± 1.7[abcd]	6.2 (6.1–6.4)	12.7 (12.3–13.3)	0.15	1319.7	<0.0001
F-ICIPE-21	90.8 ± 2[bcde]	4.2 (4.1–4.3)	11.2 (10.7–11.7)	0.10	1039.6	<0.0001
G-ICIPE-60	91.7 ± 2.1[abcde]	4.8 (4.7–4.9)	10.1 (9.7–10.5)	0.13	1269	<0.0001
H-ICIPE-30	100 ± 0[a]	4.1 (4.0–4.2)	7.7 (7.5–8.0)	0.14	1253.5	<0.0001
I-KR-16	100 ± 0[a]	5.0 (4.9–5.0)	8.7 (8.4–8.9)	0.17	1392.1	<0.0001
J-Sudan-4	80.2 ± 3.5[e]	6.8 (7.0–7.3)	15.7 (16.0–18.1)	0.16	1172.2	<0.0001
K-Caterpillar	76.8 ± 4.5[f]	6.0 (6.2–6.8)	14.8 (14.9–16.3)	0.11	1027.3	<0.0001
M-ICIPE-62	98.3 ± 1.1[ab]	4.5 (4.4–4.6)	9.6 (9.3–10.0)	0.13	1248.6	<0.0001
N-ICIPE-20	91.7 ± 3.1[abcde]	3.7 (3.6–3.9)	10.2 (9.7–10.7)	0.10	943.4	<0.0001
O-Sandfly-18	97.5 ± 1.7[abc]	3.7 (3.6–3.8)	8.3 (8.0–8.6)	0.11	1067.3	<0.0001
P-EMB-26	100 ± 0[a]	5.0 (4.4–4.6)	7.6 (7.4–7.8)	0.17	1347.4	<0.0001
R-R1/RA	88.3 ± 3.8[de]	7.6 (7.4–7.7)	15.5 (14.8–16.4)	0.18	1132	<0.0001
B. bassiana						
L-Mbita	90 ± 3.7[bcde]	5.0 (4.9–5.1)	11.6 (11.1–12.1)	0.12	1218.2	<0.0001
Q-GPK	89.4 ± 3.8[de]	5.7 (5.5–5.8)	13.3 (13.9–15.4)	0.12	1192.2	<0.0001
S-Kericho	96.7 ± 1.7[abcd]	5.6 (5.4–5.7)	13.2 (12.6–13.9)	0.12	1226.8	<0.0001

Values with similar superscript alphabets do not differ significantly; Figures in parentheses indicate 95% fiducial limits.

were harvested by sifting the substrate through a sieve (295 µm mesh size) and were stored at 4–6°C before being used in the experiments.

Inoculation of adult sandflies

Adult sandflies were contaminated using the technique described by Maniania[14] and Migiro *et al*[15]. Briefly, flies were exposed to 0.1 g of dry conidia evenly spread on a cotton velvet cloth covering the inner side of a cylindrical plastic tube (95 × 48 mm diam). In all 25 sandflies were transferred to the tube and allowed to walk on the velvet for one minute after which 20 sandflies were transferred to clean ventilated Plexiglass cages (150 × 150 × 200 mm). The sandflies were maintained on apple slices which supplied them with sugar as source of energy. Control sandflies were exposed to fungus-free velvet material before being transferred to similar ventilated Plexiglas cages. Each treatment consisted of 20 sandflies per replicate with four replications per isolate. Test sandflies were maintained at ambient conditions (25 ± 2°C and 60–70% RH). The remaining 5 sandflies were used to estimate the number of conidia picked up by a single sandfly in each treatment. Insects were transferred individually to 2 ml cryogenic tubes containing 1 ml of sterile distilled water plus 0.05% Triton X-100. The tube was vortexed for 2–3 min to dislodge conidia from the insect and the concentration of conidia was determined using a haemocytometer. Mortality was recorded daily until all the sandflies died. Dead sandflies were surface-sterilized in 70% alcohol followed by 3 rinses in sterile distilled water and transferred to Petri dishes lined with damp sterilized filter paper to promote fungal growth on the surface of the cadaver. Mycosis was confirmed by microscopic examination. Isolates that caused mortalities between 97.5 and 100% were considered to be highly pathogenic and can be used for field experiments to control sandflies.

Statistical analysis

Sandfly mortality was adjusted for control mortality using Abbott's formula[16]. Analysis of variance (ANOVA) was performed on the percentage mortality data (transformed to arcsine scale to normalize the variance). LT_{90} and LT_{50} values were determined for each replicate using the probit method. All analyses were carried out using the SAS (1990) software package.

RESULTS

Results on the susceptibility of adult sandflies are shown in Table 1. Mortality in the controls was approximately 16.8% with a standard error (S.E.) of 1.7%. Adult sandflies were susceptible to fungal infection with mortalities ranging from 76.8–100% in 12 days post-treatment. The LT_{50} varied from 3.0 to 7.8 days and LT_{90} from 5.3 to 16.2 days on all the fungal isolates (Table 1). The most virulent isolates caused mortalities ranging from 97.5–100% within 5.3–9.6 days (Table 1). There were four isolates from *M. anisopliae* group that recorded mortalities of 100%. The most virulent isolates also took fewer days to kill sandflies in the whole experiment than the less virulent ones. The poorest performer of all the isolates in our group of isolates had a mortality rate of 76.8% and took 14.8 days to reach LT_{90}. In this experiment, the best performance by *Beauveria bassiana* isolates was 96.7%.

DISCUSSION

All the 16 isolates of *M. anisopliae* and 3 of *B. bassiana* were pathogenic to adult *P. duboscqi* in the laboratory bioassays. These results are in agreement with reports of Warburg[17] on the susceptibility of *P. papatasi*, a similar species to *P. duboscqi* which is common in the Mediterranean region and the middle-east countries. Although these isolates were quite pathogenic to *P. duboscqi*, it was evident that they had varying levels of pathogenic activity. The least virulent isolate, Caterpillar, caused mortality of 76.8% while the most virulent, MER-10, ICIPE-30, KR-16 and EMB-26, induced mortality of 100%. The performance of *M. anisopliae* was better than *B. bassiana* in these laboratory-based studies on *P. duboscqi* sandflies based on mortalities of sandflies which went up to 100% on four of the *M. anisopliae* isolates.

In the study, sandflies were infected through velvet material contaminated with dry conidia. The number of conidia picked by a single sandfly was determined and varied between ($4.2 × 10^5$ and $1.0 × 10^6$ conidia per sandfly, which is within the range reported by Dimbi *et al*[18] with fruit flies. The LT_{50} and LT_{90} of the most virulent isolates ranged from 3.0–5.0 and 5.3–8.7 days, respectively. The isolate identified with letter 'O' in the study and bearing the word 'Sandfly 18' came from a soil sample collected from a sandfly habitat. We did not isolate any fungi from individual sandflies even though it had been claimed that entomopathogenic fungi are more virulent to insect species from which they were isolated[18].

The habitats of sandfly vectors in Kenya are predominantly termite mounds, animal burrows, caves and rock crevices[19-21] and may offer ideal conditions and targets for application of conidia of entomopathogenic fungi in a field setting. Encouraging results with entomopathogenic fungi were achieved in infecting and killing adult African malaria vector *Anopheles gambiae* (Diptera: Culicidae)

through tarsal contacts in the laboratory and rural African village houses[22]. The high potency of these entomopathogenic fungi in infecting and killing phlebotomine sandflies suggests that they could be the best alternative for sandfly control when compared to other forms of vector control.

The results of this study showed that six isolates identifiable as MER-10, ICIPE-30, KR-16, EMB-26, ICIPE 62 and Sandfly-18, could be ideal candidates for use as microbial control of phlebotomine sandflies based on their performance in the laboratory trials. Unlike most other pathogens of insects, the fungi penetrate via the host cuticle. This rare characteristic makes them especially important in the control of biting arthropods like sandflies. No reports of resistance have been documented on entompathogenic fungi, a factor that makes them quite suitable for the control of medically important insects. More studies are now required to test these most virulent isolates and determine effects of environmental factors on their viability and infectivity in the field. The most successful isolate can be commercially produced for use in suppressing sandfly populations in the field.

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