Pathogenicity of Ethiopian Isolates of *Metarhizium anisopliae* and *Beauveria bassiana* against the Tsetse Fly, *Glossina morsitans morsitans*

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Abstract

Entomopathogenic fungi, *M. anisopliae* EE, *M. anisopliae* MM, *B. bassiana* FF, *B. bassiana* GG, and *B. bassiana* AK isolated from different sources in Ethiopia were evaluated against the tsetse fly, *Glossina morsitans morsitans* in the laboratory. *M. anisopliae* isolates EE and MM caused mortalities of 96.67% and 73.33%, respectively while *B. bassiana* isolates coded as FF, GG, and AK showed percent moralities of 75.00%, 63.33% and 53.33%, respectively. *B. bassiana* FF was significantly better than *B. bassiana* AK ($P<0.05$). Spore production of presumably promising isolates, *M. anisopliae* MM and EE, was determined on solid substrates, whole grains of rice, wheat, barley and sorghum. Both isolates grew best on rice giving a yield of 1.42×10^9 spores/gram of rice for *M. anisopliae* MM and 1.62×10^9 spore/ gram of rice for *M. anisopliae* EE. No relationship was observed between moisture content of grain types and spore yield ($P > 0.05$). The potential of the isolates for the control of tsetse flies is discussed.

Introduction

Tsetse flies, *Glossina* spp. (Diptera: Glossindae), being vectors of African trypanosomoiasis, are of great economic and medical importance in Africa. Africa's low livestock productivity is mainly ascribed to the widespread occurrence of trypanosomiasis. Annual losses in agricultural production are estimated to be about £3 billion. More than 100 people and about 10,000 cattle die daily due to African trypanosomiasis (Hursey 2001).

Tsetse fly control since its beginning in the early part of the last century has involved many methods such as game destruction, vegetation clearance, and large-scale application of insecticides. All are considered to be ecologically damaging. Relatively recently other techniques, such as the use of traps, insecticide impregnated screens and systemic insecticide for livestock have been employed. However, no single method is entirely satisfactory (Nantulya 1986).

Although a number of natural enemies have been considered for use in an integrated control program for tsetse flies (Hamon et al. 1977) none have been tested in the field to date. Entomopathogenic fungi have shown great promise for use against tsetse flies.

In laboratory experiments, *B. bassiana* and *M. anisopliae*, have been shown to be highly pathogenic to *G. m. morsitans* (Kaaya 1989, Maniania 1994). Horizontal transmission of both entomopathogenic fungi from infected flies to non-infected ones was also demonstrated (Kaaya & Okech 1990). When larvae pupated in sand contaminated with spores of the pathogens, the emerging adults were infected (Kaaya & Munyunyi 1995). These, and more recent findings demonstrating the potential use of auto-inoculative devices to infect adult tsetse flies in the field (Maniania 1994, 1998, 2002), have improved the prospects for the successful use of entomopathogenic fungi in the tsetse control operations. The objective of this study was to evaluate Ethiopian isolates of *M. anisopliae*, and *B. bassiana* against *G. m. morsitans* and determine the potential to mass produce the isolates on locally available solid grain substrates.

Materials and Methods

Insects

Tsetse flies, *G. m. morsitans* were obtained from the insectary in the Vector Biology Laboratory, Ethiopian Health and Nutrition Research Institute. The flies were kept at temperature of 25° C and 70% relative humidity in cages made of plastic containers covered with white mesh. They were fed daily on rabbits except on Sundays and holidays.

Entomopathogenic fungi

Three *B. bassiana* isolates, coded as FF, GG, and AK and one *M. anisopliae* isolate coded as EE, were obtained from the culture collections of the Mycology Laboratory, Biology Department, Addis Ababa University. The original hosts of the different isolates are given in Table 1. An additional *M. anisopliae* isolate MM, was isolated from soil taken from *Arba Minch*, in southern Ethiopia, an area known to harbor tsetse flies.

Table 1. Original hosts of the different isolates of *M. anisopliae* and *B. bassiana*

Isolate	Insect source
M. anisopliae (EE)	Crustacean
B. bassiana (FF	Coleoptera
B. bassiana (GG)	Coleoptera
B. bassiana (AK)	Coleoptera

Isolation of fungus from soil collected in *Arbaminch*

Soil samples were collected in plastic bags from the *Arba Minch* area, southern Ethiopia, where tsetse flies are known to occur. In the laboratory, 1 gram of soil was suspended in 9 ml of sterile distilled water containing 0.2% v/v Tween 20 and shaken vigorously. The suspension was further diluted to 10^{-2} and 10^{-3} in sterile distilled water. A 0.1 ml aliquot from each dilution was plated onto Sabouraud dextrose agar (SDA). Colonies developing on the medium that appeared to be *Beauveria* or *Metarhizium* spp. were then selected and sub-cultured.

Production of inoculum for insect assays

Test fungi were grown on SDA plates at 28°C for 15 days. Spores were harvested by flooding the plates with sterile distilled water containing 0.1% (v/v) Tween-20 and scraping the spores from the surface of the medium with a spatula. The resulting suspension was filtered through sterile cotton wool to remove hyphal fragments. Spore concentration was determined using an Improved Neubauer haemocytometer and adjusted to 1 x 10^8 spores /ml for evaluation against *G. m. morsitans*.

Evaluation of efficacy

Each fungal isolate was screened against 3 replicate of 20 male flies using methods modified from those described by Kaaya (1989). Spore suspensions (10^8/ml) were prepared in 0.1% Tween-20 and 10 ml of each suspension was held in a 50 ml round-bottomed flask. Twenty chilled flies (1°C for 7 minutes) were introduced into each flask. Flasks were gently shaken for 15 seconds, after which the contents were emptied through a

piece of gauze, leaving the chilled flies on the gauze. Treated batches of flies were then transferred to separate cages. Control batches were immersed in sterile 0.1% Tween-20 only. Caged flies were held at 25°C, 70% RH for 20 days and mortality was recorded daily. Flies were fed during the assay. Few dead flies from the experimental group were checked for infection by the pathogens. This was done by surface sterilizing the dead fly by dipping them first in absolute ethanol, and then in 5% sodium hypochlorite before they are rinsed in sterile distilled water for few seconds. Finally the flies were put on SDA plates and incubated at (28°C) till fungal growth was eminent on the flies.

Production of isolates on grain substrates

M. anisopliae isolates MM and EE were cultured on rice, wheat, barley and sorghum. Seventy-five grams of each grain type in duplicates were soaked overnight in 100 ml of water in 250 ml Erlenmeyer flasks. Two replicate flasks per grain type were prepared. After discarding the excess water, the grains were autoclaved at 120°C for 30 minutes. The substrates were inoculated with 1.25 ml of spore suspension containing 10^7spores/ml of *M. anisopliae*. The flasks were held at 25 °C and 70% RH for 20 days, and were turned for a few minutes daily to promote uniform growth throughout the substrate.

Spore yield was determined by taking a 2 g sample from each flask and transferring them to a 50 ml flask containing 10 ml of sterile distilled water with 2% v/v Tween-20. The flasks were shaken to dislodge spores from the grains. One ml of the spore suspension was transferred to a vial and agitated using a vortex shaker for 6 minutes to disrupt spore clumps. Spore concentration was determined using a haemocytometer.

Moisture content of soaked grains was determined by soaking 15 g of each grain type in 20 ml of water in a 50 ml flask overnight. After pouring off the excess water, 5 g samples were taken fromeach flask and dried in an oven (80°C) until a constant weight was obtained. The weight of the dry samples was recorded to determine the percentage moisture content of the initial grain substrate.

Statistical Analysis

Data were analyzed using a student's t-test at 95% of confidence level. SPSS 10.0 software was used for the analysis. All graphs were plotted using Microsoft Excel 2000.

Results

All the five fungus isolates tested incurred mortality ranging from 53.33% (by *B. bassiana* AK) to 96.67% (by *M. anisopliae* EE) against G. *m. morsitans* by day 20 (Table 2). Among the five isolates, *M. anisopliae* EE, in addition to causing highest cumulative mortality, was found to be the most virulent achieving its maximum performance in 15 days. While the rest isolates needed five more days to achieve their maximum performance, which was still less than that of *M. anisopliae* EE.

Table 2. Mean cumulative percent mortality in adult male *G. m. morsitans* following treatment with different isolates of *M. anisopliae* and *B. bassiana* (1x10^8 conidia/ml) 5, 10, 15 and 20 days after exposure.

Isolate	Day 5	Day 10	Day 15	Day 20
M. anisopliae (EE)	21.67	78.33	96.67	96.67[a*]
M. anisopliae (MM)	10.00	38.33	65.00	73.33[ab]
Control	0.00	0.00	3.33	8.33
B. bassiana (FF)	6.67	51.67	61.67	75.00[ab]
B. bassiana (GG)	6.67	30.00	60.00	63.33[bc]
B. bassiana (AK)	1.67	23.33	43.33	53.33[c]
Control	0.00	0.00	0.00	0.00

Means of 3 cages of 20 flies are presented.
** Same letters indicate no significant difference at 5% level of confidence.*

Both isolates, *M. anisopliae* MM and *M. anisopliae* EE, grew and conidiated readily on all of the grains tested (Fig. 1). However, rice appeared to be the best substrate, yielding about 10 times more spores than rest of the substrates. Spore yield for *M. anisopliae* MM was highest on rice (1.42X10^9spores/g), followed by sorghum, wheat, and barely, respectively. Similar trends were observed for *M. anisopliae* EE (1.62x10^9 spores/g). The difference between rice and the rest of the grains was significant, $P < 0.05$ for *M. anisopliae* MM and $P < 0.01$ for *M. anisopliae* EE.

The difference in yield obtained on wheat, barley and sorghum was not significant for *M. anisopliae* EE ($P > 0.05$). The same was true for *M. anisopliae* MM except the yield of barley which was significantly less than that of sorghum ($P < 0.05$).

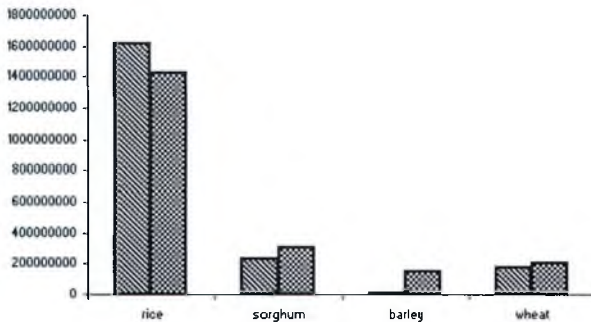

Fig 1. Mean spore yield (spores/g of substrate) of *M. anispoliae* EE and *M. anisopliae* MM on different grains.

Fig 2. Mean spore yield (spores/g of substrates) of *M. anisopliae* (EE) and percent moisture content of grains.

Discussion

The present study complements previous trials by showing that *M. anisopliae* and *B. basiana* can infect and kill tsetse flies, but there is considerable variation in the relative virulence of different fungal isolates. Poinar et al. (1977) obtained mortality levels of only 30% from these fungal species, whereas Kaaya (1989) and Kaaya & Munyunyi (1995) reported mortality rates ranging from 59.70 to 95.45% at 2.0×10^{7} spores per ml 18 days after treatment.

Compared with results obtained by Kaaya (1989) and Kaaya & Munyinyi (1995), the virulence of the isolates used in the present study seems to be relatively low, especially as they used a spore concentration that was five times lower than that used in the current study. However, direct comparisons of results of different studies unless done at the same time using identical test methods is difficult, hence the differences observed in virulence can not be taken for granted. These different results are used to highlight potential differences in virulence (Clarkson 1996, Goettel & Roberts 1992), and the fact that the isolates used in the current study at least appeared to be as virulent, or perhaps more so, than some of those previously studied.

Some investigators have tried to relate the pathogenicity of an isolate to the original host it was isolated from; i.e., strains of a species isolated from a specific insect host are more virulent for that host than strains isolated from a taxonomically unrelated host (Tanada & Kaya 1993). On the other hand, Feng & Johnson (1990) reported that *B. bassiana* strains isolated from taxonomically unrelated hosts were more virulent than the strains that were isolated from a taxonomically related host. In this study all the *Beauveria* isolates were from coleoptera, different order, and *M. anisopliae* EE was from a crustacean, different class. Since no isolate was obtained from tsetse fly in this study it is difficult to substantiate or refute the above statements.

From the present and previous studies (Kaaya 1989, Kaaya & Okech 1990, Kaaya & Munyinyi 1995, Maniania 1998, 2002), tsetse flies can be considered as viable targets for fungal control. Furthermore, the ecology of tsetse flies seems to be favorable for the use of fungal pathogens due to their preference for higher humidity's (60-85%) (Itard & Jordan 1977), which is appropriate for insect infection and sporulation (Ramosk 1984) and development of fungal epizootics (Ferron 1978). For riverine tsetse flies that inhabit waterside vegetation, where the ambient relative humidity can reach 100%, there is a

be favorable for the use of fungal pathogens due to their preference for higher humidity's (60-85%) (Itard & Jordan 1977), which is appropriate for insect infection and sporulation (Ramosk 1984) and development of fungal epizootics (Ferron 1978). For riverine tsetse flies that inhabit waterside vegetation, where the ambient relative humidity can reach 100%, there is a strong possibility that epizootics could be initiated (Kaaya 1989). It is now known that fungal pathogens can infect and kill at very low humidity's though the development of epizootics is unlikely (Bateman et al. 1993, Ramosk 1984).

Production of conidia by both *M. anisopliae* isolates was highest on rice (Fig.1). Rice is well known for being a very suitable material for production of *M. anisopliae* and *B. bassiana* compared to other substrates (Vilas Boas 1996, Ibrahim & Low 1993) and it is widely used in the mass production of *B. bassiana* and *M. anisopliae* (Mendonca 1992, Goettel & Roberts 1992). The highest spore yield obtained in our studies was 1.62×10^9 spores/g of rice. Gitonga (1996) reported a yield of 1.25 x 109 spores/ g of rice and Mendonca (1992) reported a yield of 1×10^{10} spores of *M. anisopliae* per gram of rice. Direct comparison of yields is though difficult due to slight differences in the methodology used for their production, and the age of the culture at harvest.

Although the moisture content of the substrate rice was the highest, there appeared to be no correlation with spore yield as barley, which gave the lowest yield, had the second highest moisture content. Gitonga (1996) also reported moisture content did not influence the quantity of *M. anisopliae* and *B. bassiana* conidia obtained from rice.

Mass production of fungi using readily available inexpensive substrates is an appropriate technology suitable for developing countries. For example, in Brazil solid substrate fermentation on rice is used to produce *M. anisopliae* for controlling sugar cane froghopper, *Mahanarva posticata* (Mendonca 1992). The present study has shown that locally isolated entomopathogenic fungi can infect and kill tsetse flies and may be readily produced on locally available substrates like rice, sorghum and wheat. Thus there is a great potential to utilize these microorganisms in an integrated tsetse fly control program. It is, however, important to develop quality control procedures that help to maximize product performance, ensure product safety, standardize cost and reduce the risks of supply failure, thus building user confidence. Another important aspect in the use of biopesticides is its application in the field. Tsetse fly is known to occupy vast areas in Africa. Proper formulation and application methods which enable to cover substantial area or devices which use the fly's behavior to disseminate the pathogen must be developed side by side with the development of the fungal pathogen production technologies before any control plan is contemplated.

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References

Bateman RP, M Carey, D Moore and C Prior. 1993. The enhanced activity of *M. flavoviride* in oil formulations to desert locusts at low humidities. Annals of Applied Biology **122:** 145-152.

Clarkson JM. 1996. Molecular biology of fungi for the control of insects, pp. 123-135. *In:* Gunasekaran M, DJ Weber and Boca Raton (eds) Molecular Biology of the Biological Control of Pests and Diseases of Plants. Florida, CRC press.

Feng MG and JB Johnson. 1990. Relative virulence of six isolates of *B. bassiana bassiana* on *Duraphis noxia* (Homoptera: Aphidae). Environmental Entomology **19:** 785-790.

Feron P. 1978. Biological control of insect pests by entomogenous fungi. Annual Review of Entomology **23**: 409-442.

Gitonga W. 1996. *M. anisopliae anisopliae* (Metschnikoff) Sorokin *and B. bassiana bassiana* (Balsamo) Vuillemin as Potential Biological Control Agents of *Macroterms michaelseni* (Sjostedt). (Isospora: Termitdae) in Kenya, Ph.D. Thesis. Department of ecology and Molecular Biology, Royal Veterinary and agricultural University Copenhagen, Denmark.

Goettel MS and RW Roberts.1992. Mass production formulation and field application of entomopathogenic fungi, pp. 230-238. *In:* Lomer CJ and C Prior(eds). Biological Control of Locusts and Grasshoppers. UK, CAB International.

Hamon J, DAT Baldry, JD ParkerA Callier and AR Stiles. 1977. Status and Future of Control, pp. 35-44. *In:* Laird M (ed) Tsetse: The future for biological methods in integrated control. Ottawa, IDRC- 077E.

Hursey BS. 2001. The Program Against African Trypanosomiasis: aims, objective and achievements. Parasitology Today **17**: 2-3.

Ibrahim YB and W Low. 1993. Potential of mass-production and field efficacy of isolates of the entomopathogenic fungi *B. bassiana bassiana* and *Paecilomyces fumosoroseus* against *Plutella xylostella*. International Journal of Pest Management**39**: 288-292.

Itard J and AM Jordan. 1977. Mass rearing using animals for feeding, pp.125-140. *In*: Laird M (ed) Tsetse: The future for Biological Methods in integrated Control. Ottawa, IDRC-O77E.

Kaaya GP. 1989. *Glossina moristans morsitans*. Mortalities caused in adults by experimental infection with enthomopathogenic fungi. Acta Tropica **46**: 107-114.

Kaaya GP and MA Okech. 1990. Horizontal transmission of mycotic infection in adult tsetse, *Glossina moristans moristans*. *Enthomophaga* **35:** 589-600.

Kaaya JP and DM Munyinyi. 1995. Biocontrol potential of the enthomopathogenic fungi *B. bassiana* and *M. anisopliae* for tsetse flies (*Glossina* spp.) at development sites. Journal of Invertebrate Pathology **66**: 237-241.

Maniania NK. 1994. Laboratory technique for infecting adult tsetse with a fungal pathogen. Insect Science Application **15**: 421-426.

Maniania NK. 1998. A device for infecting adult tsetse flies *Glosina* spp with an entomopathogenic fungus in the field. Biological Control **11**: 248-254.

Maniania NK. 2002. A low cost contamination device for infecting adult tsetse flies, *Glosina* spp., with the entomopathogenic fungus *Metarhizium anisopliae* in the field. Biological Control Science and Technology.In press.

Mendonca AF. 1992. Mass production, application and formulation of *Metarhizium anisopliae* for the control of sugarcane froghopper, *Mahanarva postica,* in Brazil, pp.239-244. *In:* Lomer CJ and C Prior (eds) Biological Control of Locusts and Grasshoppers. UK, CAB International.

Nantulya VM. 1986. Immunological approaches to the control of animal trypanosomiasis. Parasitology Today **2:** 168-173.

Poinar JrJO, L Van der Geest, W Helle and H Wassink. 1977. Pathology and nematode parasitism, pp. 75-92. *In:* Laird M (ed) Tsetse: The Future for Biological Methods in Integrated Control. Ottawa, IDRC-077E.

Ramosk WA. 1984. The influence of relative humidity on *B. bassiana bassiana* infectivity and replication in the chinch bug, *Blissus leucopterus.* Journal of Invertebrate Pathology **43**: 89-394.

Tanada Y and HK Kaya. 1993. Insect pathology. San Diego: Academic Press. pp. 666.

Vilas Boas AM, RM Andrade and JV Oliveira. 1996. Diversification of culture media for production of entomopathogenic fungi. Arquivos de Biologiae Technologia **39**: 123-128.