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Potential of some *Metarhizium anisopliae* isolates for control of *Culex quinquefasciatus* (Dipt., Culicidae)

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Abstract: The potential of some isolates of *Metarhizium anisopliae* and *Beauveria bassiana* for use in the integrated management of *Culex quinquefasciatus* was evaluated. *Metarhizium anisopliae* isolate 1037 was selected in initial bioassays with a 50% lethal concentration (LC_{50}) of 1.97×10^4 conidia/ml. This fungus caused higher mosquito larva mortality when applied as a conidial suspension to the surface of the water than as dry conidia, with a time to 50% lethal (LT_{50}) of 1 day compared with 3.6 days for the dry conidial application. However, results with UV- and heat-inactivated conidia did not confirm a possible role of fungal toxins in causing mortality when ingested by *C. quinquefasciatus* larvae. *Metarhizium anisopliae* did not remain active for as long as the bacterium *Bacillus sphaericus* isolate 2362. At 24 h after application, the fungus-induced mortality on mosquito larvae was significantly lower than the mortality caused by the bacterium. By the second day, almost no activity by the fungus was observed. Results suggest that *M. anisopliae* isolate 1037 has potential for use in mosquito control programmes.

1 Introduction

Human interference in the environment often results in changes in the equilibria among the species in different systems. The accumulation of organic matter, especially due to the misplacement of household garbage and other waste materials, changes in the topography and in the drainage systems favour certain mosquito species. These species become anthropophilic and gain importance as vectors of human diseases. Species of the genus *Culex* are among those that have adapted well to habitats created by human interference. *Culex quinquefasciatus* Say (Dipt., Culicidae) is the vector of human filariasis (brancroftian filariasis) in several locations including the north and north-east regions of Brazil. In other countries in North and South America, Africa, and Asia, this species has also been implicated as a vector of protozoan and viral pathogens of birds and equines (Charlwood, 1979; Cheng, 1986; Regis et al., 1995).

Around the world, the use of chemical insecticides is still the most important element in mosquito control programmes. However, since the 1970s several cases of resistant mosquito populations have been reported (Cônsoli et al., 1986). Most control strategies target the adult insects, such as ULV fogging with low residual products or the application of contact pesticides to mosquito resting areas. Application strategies targeting mosquito larvae are increasingly using insect growth regulators (IGRs), *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. In addition to the problems with resistant populations, chemical pesticides also cause other serious problems, such as indirect and direct toxic effects on non-target organisms including humans. The environmental disequilibria caused by these products may also cause the elimination of beneficial organisms, which can lead to higher populations of pestiferous mosquitoes.

Human communities affected by mosquitoes and their control measures must be consulted and involved in mosquito control strategies. Such strategies, must be researched and implemented, and should be based on an integrated pest management approach, including biological, chemical and physical control methods (Regis et al., 1995). Certain entomopathogens, such as the fungi *Lagenidium giganteum* Couch (Oomycetes), *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Tolypocladium* sp., and *Culicinomyces* (Deuteromycetes), and especially bacteria from the genus *Bacillus*, have great potential for use in the integrated control of mosquitoes. Such pathogens are especially interesting due to the possibility of low cost *in vitro* production and the high virulence shown by some subspecies or isolates (Roberts, 1970; Lacey et al., 1988; de Barjac and Sutherland, 1990; Sandhu et al., 1993).

Studies on the isolation and selection of new isolates of these entomopathogens are important because of the great natural genetic variation that exists in these microorganisms. Studies in this area can provide isolates that will greatly improve the efficacy of microbial control strategies in the field. Greater production of infective units, higher virulence and better survival of infective units in the environment (resistance to UV radiation, high temperatures and

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adverse chemical conditions) are some of the factors which can result in more efficient microbial control.

Past research on the fungus *M. anisopliae* has evaluated the potential of this agent for the control of mosquitoes, taking into consideration its mode of action, effects of water quality, storage and formulation of conidia (Roberts, 1970; Ramoska et al., 1981; Daoust et al., 1982; Lacey et al., 1988). The research reported herein tested the potential of some isolates of *M. anisopliae* for use in the integrated management of *C. quinquefasciatus*.

2 Materials and methods

2.1 Insects

Adult mosquitoes were maintained in cages (70 cm × 50 cm × 50 cm) and fed a 10% dilution of honey in water. In addition, blood was offered to females three times a week by placing a mouse in the cage. Plastic cups with water were placed in the cages to serve as oviposition sites. The eggs masses were collected daily and maintained in plastic cups until egg eclosion. The larvae were reared in plastic pans and were fed ground rodent chow as needed. The pupae were collected and transferred to new cups and the adults were allowed to emerge inside the cages described above.

2.2 Fungal isolates

Brazilian isolates of the fungi *M. anisopliae* and *Beauveria bassiana* (Balsamo) Vuillemin were obtained from the entomopathogen collection maintained at the Laboratory of Insect Pathology and Microbial Control at the Entomology, Phytopathology, and Zoology Department of the Escola Superior de Agricultura 'Luiz de Queiroz', University of São Paulo, Brazil. Isolates were initially selected from the collection based on survival in storage. Plates from routine viability tests were used as the initial inoculum for experiments.

Fungal isolates were stored on both potato dextrose agar (PDA) covered with mineral oil at 10°C, and as pure dry conidia at −15°C. Fungal cultures were obtained by inoculating fresh PDA plates which were incubated at 26°C and a 12 h photophase for 7–10 days. After fungal sporulation, the conidia were collected by scraping the surface of the medium with a rubber spatula, and saved for future use as described above.

2.3 Isolate selection and virulence comparison

Six *M. anisopliae* and two *B. bassiana* isolates were used in initial isolate selection (table 1). Isolate E9 (our standard treatment) has been used in Brazil extensively in both experimental and commercial preparations, especially for the control of spittlebugs in sugarcane and pastures. One gram of conidia from each isolate was suspended in distilled water + 0.1% Tween 80. Conidial concentrations were determined using a haemocytometer and phase contrast microscope. Dilutions containing 2×10^8 conidia/ml were prepared by adding water to original suspensions as needed. These suspensions (1 ml) were added to each bioassay cup containing 99 ml of distilled water for a final suspension of 2×10^6 conidia/ml bioassay water. Fifteen third-instar *C. quinquefasciatus* larvae were added to each of four cups (replicates) used per treatment.

After adding the larvae to the cups, a pinch of ground rodent chow was added to the water as food for the insects, and the cups were maintained at 26°C and 12 h photophase. Evaluations of larval mortality were carried out after 24 and 48 h when dead larvae, defined as those not reacting when touched, were removed.

2.4 Virulence comparison

The virulence of the isolates 1037 and E9 were compared using seven conidial suspensions in water (7.85×10^3, 1.57×10^4, 3.15×10^4, 6.25×10^4, 1.25×10^5, 2.50×10^5, 5.00×10^5 conidia/ml). Four replicates of each treatment were prepared in cups containing 200 ml of distilled water to which 15 third-instar *C. quinquefasciatus* larvae were added. The insects were fed and maintained as described above at 25 ± 1°C. Mortality was evaluated daily for 7 days, and the concentration necessary to kill 50% of the exposed population (LC_{50}) was estimated for each isolate using Polo-PC (Russell et al., 1977).

2.5 Application and persistence test

Isolate 1037 was used in tests to determine the viability of the conidia after addition to water. Aquaria, each containing 30 third-instar *C. quinquefasciatus* larvae were treated in one of three ways: (a) dry conidia applied to the water surface; (b) conidial suspension in 0.1% Tween 80 dripped onto the water surface; (c) 0.1% Tween 80 in distilled water (control). The final fungal concentration in the aquaria was 10^8 conidia/ml. Three aquaria were used for each treatment for a total of 90 larvae per treatment. Dead larvae were removed daily for 7 days, and the time necessary to reach 50%

Table 1. *Entomopathogenic fungal isolates used in bioassays against* Culex quinquefasciatus *larvae*

Isolate[a]	Original host or substrate	Place of origin (City – State)
Metarhizium anisopliae var. *minor*		
1189	soil	Corumbá – MS
Sul-1	(unknown)	(unknown)
412	*Epilampra* sp. (Blaberidae)	Iguape – SP
1184	soil	Corumbá – MS
E9	*Deois flavopicta* (Cercopidae)	Vitória – ES
1037	*Solenopsis invicta* (Formicidae)	Porto Alegre – RS
Beauveria bassiana		
447	*Solenopsis invicta*	Cuiabá – MT
1202	*Blattella germanica* (Blattellidae)	Piracicaba – SP

[a] Numbers represent ESALQ entomopathogen collection reference numbers.

mortality (LT_{50}) was estimated using Polo-PC. Analysis of variance and Fisher's protected least significant difference were used to compare mean mortalities among treatments.

The viability of conidia recovered from the aquaria was evaluated by plating water samples on PDA plates. Plates were incubated at 26°C for 14–16 h before germination of conidia was determined using a phase-contrast microscope to count germinated and ungerminated conidia.

2.6 Identification of source of virulence

This test was designed to evaluate whether the virulence associated with conidia was due to exotoxins and/or thermostable enzymes, or to direct fungal infection. Conidia of isolate 1037 were killed by exposure to heating (50°C), or to ultraviolet radiation (254 nm, at a distance of 20 cm from the radiation source) for 48 h. In both cases, 0.5 g of conidia were added to each of 20 Petri dishes (100 mm in diameter). The plates were agitated then inverted to eliminate conidia which did not adhere to the plate. After exposure to heat or UV radiation, the conidia were maintained in a refrigerator at 4°C until used in experiments. The lack of viability was confirmed on exposed conidia by plating on PDA plates, and the viability was determined as described above. Only batches with 0% germination after incubation for 96 h at 26°C were used. Conidial suspensions containing 3.15×10^6, 1.25×10^7 and 1.00×10^8 conidia/ml in distilled water + 0.1% Tween 80 were prepared with killed conidia. These suspensions were used in an experiment similar to that described in Section 2.4 above.

2.7 Comparison between *M. anisopliae* 1037 and *B. sphaericus* 2362

An aquarium experiment as described above for the application and persistence test was used to compare the efficacy and persistence of *M. anisopliae* (isolate 1037) with that of the bacterium *B. sphaericus* Neide (isolate 2362). Both pathogens were applied at a rate of 1×10^8 conidia or colony-forming units/ml of water in the aquaria. For the following 7 days, 30 third-instar *C. quinquefasciatus* larvae were added daily to each aquarium, after removal of the larvae added on previous day. The surviving larvae removed from the aquaria were transferred to cups with clean water and observed for an additional 24 h. The total percentage mortality of larval batches added daily to the experimental aquaria were obtained by adding mortality that occurred in the aquaria (first day) and in the plastic cups (second day).

3 Results and discussion

3.1 Isolate selection and virulence comparison

The percentage mortality of *C. quinquefasciatus* larvae treated with the different fungal strains varied from 0 to 93%, with most isolates causing very low mortality (< 20%) (table 2). Only isolate 1037 caused mortality above 90%. These results are similar to those by Daoust and Roberts (1982) who found only 30% of tested isolates causing mortality > 90%. Isolate 1037 is also highly virulent against other insects such as the sugarcane borer (*Diatraea saccharalis*), termites (*Cornitermes cumulans*), and nymphs of the German cockroach (*Blattella germanica*) (Alves et al., 1997). This isolate causes high mortality rates in a shorter time than other isolates.

Table 2. *Percentage mortality of* Culex quinquefasciatus *larvae 5 days after treatment with* Metarhizium anisopliae *and* Beauveria bassiana *isolates*

	Percentage mortality[a]	
Isolate	Mean	SEM
Metarhizium anisopliae var. *anisopliae*		
1189	10.00	7.94 cd
Sul-1	16.67	6.94 cd
412	0.00	– d
1184	18.33	3.19 c
E9	55.55	5.69 b
1037	93.33	2.72 a
Beauveria bassiana		
447	5.00	3.19 cd
1202	3.33	3.33 d
Control	0.00	– d

[a] Means followed by the same letter are not significantly different (Fisher's protected least significant difference at 5% level).

3.2 Virulence comparison

Equivalent doses of isolates E9 and 1037 resulted in very different levels of mosquito mortalities (fig. 1). For most doses, isolate 1037 caused more than twice the *C. quinquefasciatus* mortality caused by isolate E9. For all except the two lowest doses, mortalities caused by isolate 1037 were significantly higher that those caused by isolate E9. Previous work with isolate E9 produced higher levels of mortality than those observed in our experiments (Daoust and Roberts, 1982; Mohamed et al., 1983). However, variations in experimental methods may explain the observed differences. In our experiments, the estimated LC_{50} was 1.97×10^4 conidia/ml for isolate 1037, compared with 3.01×10^5 for isolate E9. Although 15 times lower than for isolate E9, the LC_{50} for isolate 1037 was higher than that calculated previously for other *M. anisopliae* isolates against a different *Culex* species (Sandhu et al., 1993). However, differences in fungal isolate activity against closely related species is a common observation. Isolate 1037 seems to be very effective against *C. quinquefasciatus*, and its use in place of E9 in control programmes is advantageous.

3.3 Application and persistence test

The application of conidial suspension to the surface of the water caused faster mortality than application of dry conidia (fig. 2). After 24 h, more than 40% of the mosquito larvae were dead in the conidial-suspension treatment, compared with 0% mortality for the dry-conidia treatment and control. The total mortality levels for the two application treatments were significantly different for the first 6 days of the experiment, but after 7 days, the difference between treatments was not significant. However, the LT_{50} values for the two application methods were significantly different. The conidial suspension had an LT_{50} of 1 day [95% confidence intervals (CI) = 0.4–1.4 days] compared with 3.6 days (95% CI = 3.0–4.2 days) for the dry conidial application. Lacey et al. (1988) obtained similar results and observed that dry conidia applied to the water

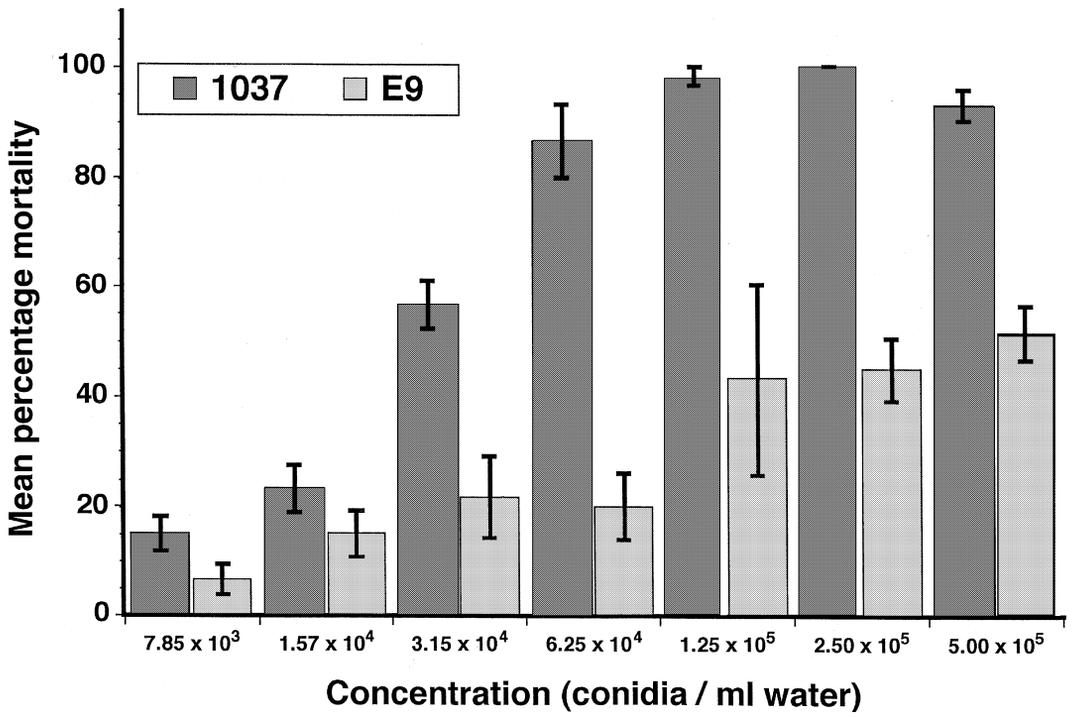

Fig. 1. *Percentage mortality of* Culex quinquefasciatus *third instar larvae exposed for 7 days to water containing different concentrations of two* Metarhizium anisopliae *isolate (1037 and E9). Bars represent ± SEM*

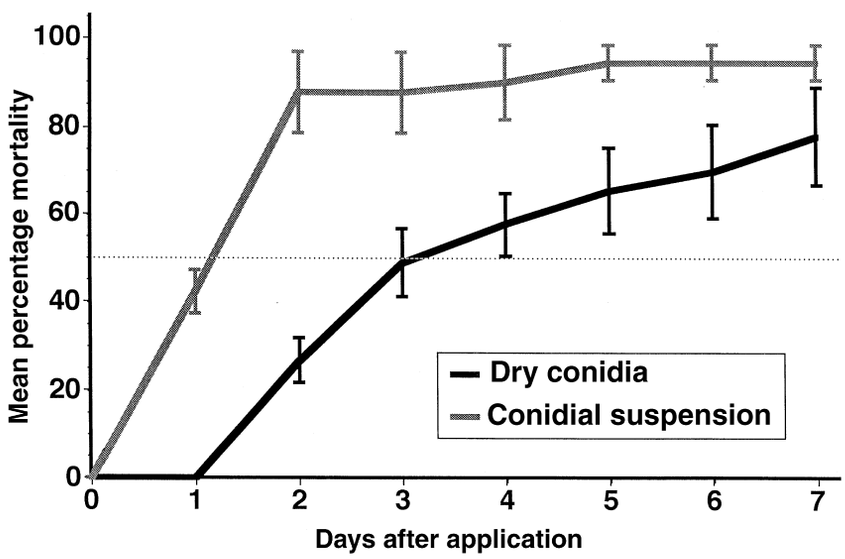

Fig. 2. *Cumulative mortality of* Culex quinquefasciatus *third instar larvae exposed for 7 days to water treated with* Metarhizium anisopliae *(isolate 1037) conidia applied either as dry powder or suspended in water. Bars represent ± SEM, and dotted line indicates 50% mortality*

surface caused mosquito mortality by germinating and growing on the respiratory apparatus of larvae. This process causes blockage of larval respiration, which is slower than the process initiated by suspended conidia in the water. The suspended conidia are ingested by the mosquito larvae and, during the digestive process, toxins and enzymes are liberated from the digested and germinating conidia in the insect gut.

Although application of conidial suspensions may cause faster mortality in laboratory experiments, the use of this technique in large-scale mosquito control programmes may not be practical. The rapid loss of conidia from the suspension caused by sinking and loss of viability, as we observed in our experiments, may favour the application of dry conidia. The application of dry conidia to the water surface may provide lower mortality but a prolonged activity against mosquito larvae (Roberts, 1970).

3.4 Identification of source of virulence

Conidia treated with heat or ultraviolet radiation did not cause mortality in *C. quinquefasciatus* larvae. This was true even when the conidia were observed in the digestive system of the larvae, as observed previously (Lacey et al., 1988). Microscopic observation showed

that some conidia were digested in the mosquito gut. However, the lack of mortality suggests that *M. anisopliae* isolate 1037 does not produce toxins at sufficient levels to cause mortality when ingested by *C. quinquefasciatus*. In addition, similar results with UV- and heat-treated conidia demonstrate that high temperatures were not responsible for inactivation of toxins. Although the presence of high toxin titres may favour insect control, it is undesirable due to safety issues related to non-target species and human exposure.

3.5 Comparison between *M. anisopliae* 1037 and *B. sphaericus* 2362

For the first day after application, the levels of mortality caused by *M. anisopliae* (isolate 1037) and *B. sphaericus* (isolate 2362) were close to 100% (table 3). However, at 24 h after application, the mortality caused by the fungus was significantly lower than that caused by the bacterium. Mortality in the fungal treatment dropped to almost 0% by the second day, whereas the bacterium continued to kill 100% of the mosquito larvae introduced to the water for 7 days. No fungal activity was observed beyond the fourth day of the experiment. Although the lack of residual activity by *M. anisopliae* lowers its insect control potential, low residual activity is desirable in some situations as in reservoirs used for drinking water. The absence of viable fungus may be a requirement before human consumption of treated water is allowed.

Further studies on the effects of water quality on *M. anisopliae* isolate 1037 and its activity against mosquito larvae are needed. However, initial results suggest that this isolate can be used in mosquito control programmes, with weekly application, alone or perhaps in combination with other control agents such as *B. sphaericus*. Alternating the use of these microbial agents may prevent the development of mosquito populations that are resistant to the bacterium (Rodcharoen and Mulla, 1994; Silva-Filha et al., 1995). Studies on this integrated approach and on the production of toxins and enzymes by the fungus *M. anisopliae* may provide important information for the future use in mosquito control.

Table 3. *Mortality (mean ± SEM) of* Culex quinquefasciatus *treated with* Metarhizium anisopliae *or* Bacillus sphaericus *in laboratory aquaria*

	Percentage mortality [a]	
Days after application	*M. anisopliae* (isolate 1037)	*B. sphaericus* (isolate 2362)
1	89.16 ± 2.50	100.00
2	2.50 ± 0.83	100.00
3	4.17 ± 2.10	100.00
4	0.00	100.00
5	0.00	100.00
6	0.00	100.00
7	0.00	100.00

[a] Results were significantly different for the two pathogens for all dates (Fisher's protected least significant difference at 5% level).

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