ARTIGO

EFFECT OF *METARHIZIUM ANISOPLIAE* ISOLATED FROM SOIL SAMPLES OF THE CENTRAL BRAZILIAN CERRADO AGAINST *AEDES AEGYPTI* LARVAE UNDER LABORATORY CONDITIONS

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ABSTRACT

The larvicidal activity of *Metarhizium anisopliae* which originated from soil samples of the Central Brazilian Cerrado was tested on *Aedes aegypti*. Mortality of second instar larvae (L2) varied between 10 and 100%, 10 days after application of 5 x 10^6 conidia/ml of 80 isolates. For some isolates, a sluggish behavior and accelerated mortality of L2 within few hours after treatment was observed. Larvae which had succumbed 24 hours after treatment were recovered with an elevated number of ungerminated conidia. A concentration of conidia was noted at the tip of the siphon and in the oral brushes of these larvae. The alimentary canal was generally replete with ungerminated conidia. Variability of initial mortality, between 24 hours and 3 days, within replicates was noted for several isolates. Values of LT_{50} varied from 1.9 (IP 23) to 3.2 (IP 42) days and LT_{90} from 2.8 (IP 84) to 7.8 (IP 76) days. Results showed that the *M. anisopliae* isolates tested, which induced quick and high mortality, have potential for biological control of *A. aegypti*.

KEYWORDS: *Aedes aegypti. Metarhizium anisopliae.* Biological control. Cerrado.

INTRODUCTION

Bioinsecticides based on bacteria, such as *Bacillus thuringiensis var. israelensis* or *B. sphaericus,* are used successfully against mosquito and blackfly larvae (9). The risk of insect resistance against not only chemicals but also

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Recebido para publicação em 6/3/2004. Revisto em 3/6/2004. Aceito em 21/6/2004.

bioinsecticides (3, 12, 26, 30), combined with an increasing concern about consequences of chemicals on the environment and human health, underline the need for further development of effective and sustainable vector control. Entomopathogenic fungi are important agents for insect pest control (14, 17, 32). However, mosquito-specific aquatic *Lagenidium giganteum*, *Culicinomyces clavisporus*, *Tolypocladium cylindrosporum* or *Coelomomyces* spp have potential but little practical importance for vector control (9), and there is only one registered product, Laginex, based on *L. giganteum* (1). By contrast *Metarhizium anisopliae* is commonly used for control of terrestrial pests (8), but there is little information about the natural occurrence of *M. anisopliae* in mosquito larvae (2). This fungus is reported to be active against aquatic stages of mosquitoes (6, 25, 28). In this study, we report the activity of *M. anisopliae* isolates which originated from the Cerrado in Central Brazil against larvae of *Aedes aegypti* under laboratory conditions.

MATERIAL AND METHODS

Origin and culture of fungi

A total of 80 isolates of *M. anisopliae* was tested. Isolates which originated from soil samples were collected in three different localities in the State of Goiás, Brazil, and were stored at the Institute of Tropical Pathology and Public Health (IPTSP), Federal University of Goiás (UFG), Goiânia, Brazil. Eight isolates were from Silvânia, 31 from the Northern region of the State and 41 isolates from the Ema National Park. All isolates were previously passaged on third instar nymphs of *Triatoma infestans* at the beginning of the reported tests. Fungi were then cultivated for 15 days at 25 ± 1°C and a photoperiod of 12:12 (L:D) hours on potato dextrose agar (PDA) medium (170 g scrubbed and sliced potatoes were boiled in 1.000 ml deionized water for 1 hour, then passed through a fine sieve and the liquid after cooling amended with 20 g glucose and 20 g agar and then sterilized at 120°C for 20 minutes). Conidia were harvested from the surface of the medium directly by scraping and then suspended in sterile 0.1% Tween 80. Suspensions were adjusted to defined concentrations based on hemacytometer counts.

Insect culture

Experiments were carried out on *A. aegypti* second instar larvae (L2). The insect population originated from virus-free adults, captured in 1998 in Goiânia and reared under laboratory conditions at the IPTSP-UFG as described by Silva et al. (29). Larvae were fed with dry triturated ration for cats.

Standard Bioassay Procedure

In a first series of experiments, 10 recently molted L2 were transferred to white plastic cups (3.5 x 4.2 cm) containing 18 ml of deionized water and incubated at 25°C during 24 hours. Then 2 ml of suspended conidia (5 x 10^7 conidia/ml) of all 80 isolates were applied in order to obtain a final concentration of 5 x 10^6 conidia/ml. Control larvae were treated with water only. Cups with larvae were then incubated at 25°C, 75% relative humidity (RH) and a photoperiod of 12:12 (L:D) hours.

A second series of experiments tested the activity of 15 isolates which had caused 100% mortality, 5 days after incubation in the previous experiments. For this, larvae and control insects were prepared and treated as mentioned before. Each treatment consisted of 4 replicates with 10 L2. Control larvae were treated with water only. Larvae were fed on alternate days as above and evaporated water in the cups was replaced daily. Mortality was recorded daily for 10 days and dead larvae were removed and examined by light microscopy. Viability of conidia was checked at each experiment. Suspended conidia of each isolate were inoculated on PDA and germination examined microscopically 24 hours after incubation at 25°C and a photoperiod of 12:12 (L:D) hours.

Analysis of data

Mortality data provided in the second experiment were arcsin-square root transformed and then analyzed using analysis of variance and the Student-Newman-Keuls (SNK) multiple range test of comparison of means (27). Means were considered not statistically different at $P > 0.05$. Probit analysis for determination of LT_{50} and LT_{90} were conducted on total mortality.

RESULTS

Larvae of *A. aegypti* were affected by all 80 *M. anisopliae* isolates tested (Table 1). Cumulative mortality ranged from 10 to 100%, 10 days after exposure to conidia; 55% and 58.8% of all isolates killed ≥ 80% of larvae within five and ten days after treatment, respectively. High rates of mortality (≥ 90%) up to 24 hours after treatment were found for 7 isolates (IP 16, IP 58, IP 76, IP 80, IP 83, IP 105 and IP 120). Larvae treated with these isolates exhibited a sluggish behavior within a few hours of exposure to conidia and either sank or floated. Microscopic examinations of L2 which had succumbed 24 hours after treatment revealed an elevated number of ungerminated conidia on the larval surface. Some conidia were distinctly swollen, but no germination-initiated conidia were observed. A concentration of conidia was noted at the tip of the siphon and on the oral brushes. The alimentary canal was generally replete with ungerminated conidia.

Table 1. Cumulative mortality (%) of second instar larvae of *Aedes aegypti* after treatment with conidia of *Metarhizium anisopliae* isolates from Central Brazil*

Isolate	Days after application			Isolate continuation	Days after application		
	1	5	10		1	5	10
IP 5	60	100	100	IP 84	50	100	100
IP 10	0	60	60	IP 85	0	10	70
IP 16	90	100	100	IP 86	10	30	50
IP 17	20	60	70	IP 87	0	70	70
IP 19	40	100	100	IP 88	0	20	20
IP 23	70	100	100	IP 90	0	20	60
IP 25	0	80	80	IP 91	0	30	80
IP 26	10	70	90	IP 92	40	80	80
IP 28	30	80	90	IP 93	0	0	70
IP 29	10	100	100	IP 96	0	80	80
IP 31	10	50	80	IP 97	10	50	80
IP 32	0	90	100	IP 101	0	50	50
IP 34	50	100	100	IP 102	30	90	100
IP 35	10	100	100	IP 103	0	20	20
IP 36	0	10	40	IP 105	90	100	100
IP 37	20	80	100	IP 106	0	0	60
IP 38	0	0	70	IP 107	60	90	100
IP 39	0	20	80	IP 108	10	60	80
IP 40	0	80	100	IP 109	0	20	100
IP 41	0	10	60	IP 110	80	100	100
IP 42	0	100	100	IP 112	0	50	50
IP 43	10	80	80	IP 113	60	90	90
IP 45	0	80	90	IP 115	40	80	90
IP 46	0	10	20	IP 116	30	40	40
IP 49	10	100	100	IP 117	80	100	100
IP 50	0	10	90	IP 119	0	40	80
IP 51	10	70	90	IP 120	90	90	100
IP 56	0	20	70	IP 123	0	30	40
IP 57	0	100	100	IP 124	0	10	70
IP 58	90	100	100	IP 125	0	20	50
IP 62	0	90	100	IP 130	50	100	100
IP 65	20	70	70	IP 134	0	0	30
IP 75	0	0	20	IP 138	0	30	50
IP 76	90	100	100	IP 139	0	0	10
IP 77	0	30	30	IP 140	20	40	80
IP 79	10	10	20	IP 143	20	40	40
IP 80	90	100	100	IP 144	30	80	90
IP 81	80	100	100	IP 145	0	40	50
IP 82	10	10	20	IP 146	0	10	20
IP 83	100	100	100	IP 151	0	0	20

*Collection of entomopathogenic fungi, Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, Brazil. Isolates originated from soil samples collected in the Cerrado of Central Brazil. IP 5 - IP 84: Ema National Park; IP 85 - IP 134: Northern region of Goiás; IP 138 - IP 151: Silvânia. One replicate of 10 individuals for each isolate at 5×10^6 conidia/ml. Mortality of control larvae < 10%, 10 days after incubation.

Further studies with 15 isolates which had killed ≥ 90% of the larvae showed no significant effect of isolates tested on mortality, 5 days ($F_{14,74} = 0.79$; $P = 0.677$) and 10 days ($F_{14,74} = 0.66$; $P = 0.825$) after application of conidia, respectively. Means of cumulated mortality found at 5 days after application are shown in Figure 1. Final mortality, 10 days after treatment, caused by the 15 isolates ranged from 75 (SEM: 13.3%) (IP 81) to 100% (IP 23, IP 84 and IP 117) (Table 2). Several isolates showed an elevated variability within the number of killed larvae, especially in the first days after treatment. Mortality did not start at the same moment in different replicates of the same isolate as shown for IP 23, IP 84, IP 117, IP 130, IP 76 and IP 81 in Figure 2. Most larvae were killed within 2 and 3 days after treatment and also showed a sluggish behavior before dying. Values of LT_{50} ranged from 1.9 (IP 23) to 3.2 (IP 42) days, and LT_{90} from 2.8 (IP 84) to 7.8 (IP 76) days, respectively. Both, LT_{50} and LT_{90}, differed significantly between isolates (Table 2). Control mortality did not exceed 5% of larvae until the end of both series of experiments. Surviving fungus-treated larvae pupated between 4 and 9 days after treatment compared to pupation of control larvae which had completed on the fourth day. Viability tests showed rates of germination > 98%, 24 hours after incubation on PDA.

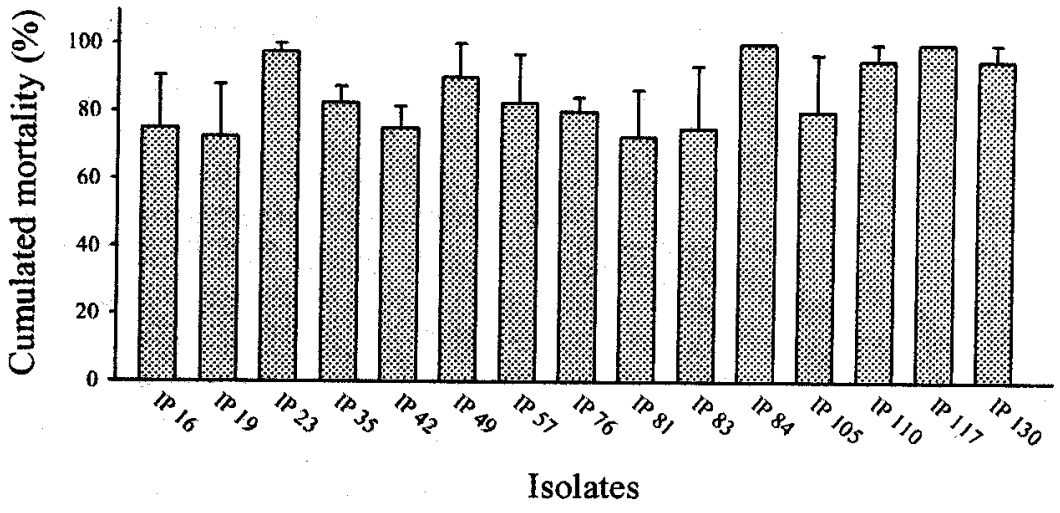

Figure 1. Cumulated mortality (means of 4 replicates of 10 individuals each with standard error of the mean) of *Aedes aegypti* second instar larvae 5 days after application of *Metarhizium anisopliae* isolates from Central Brazil at 5 x 10^6 conidia/ml

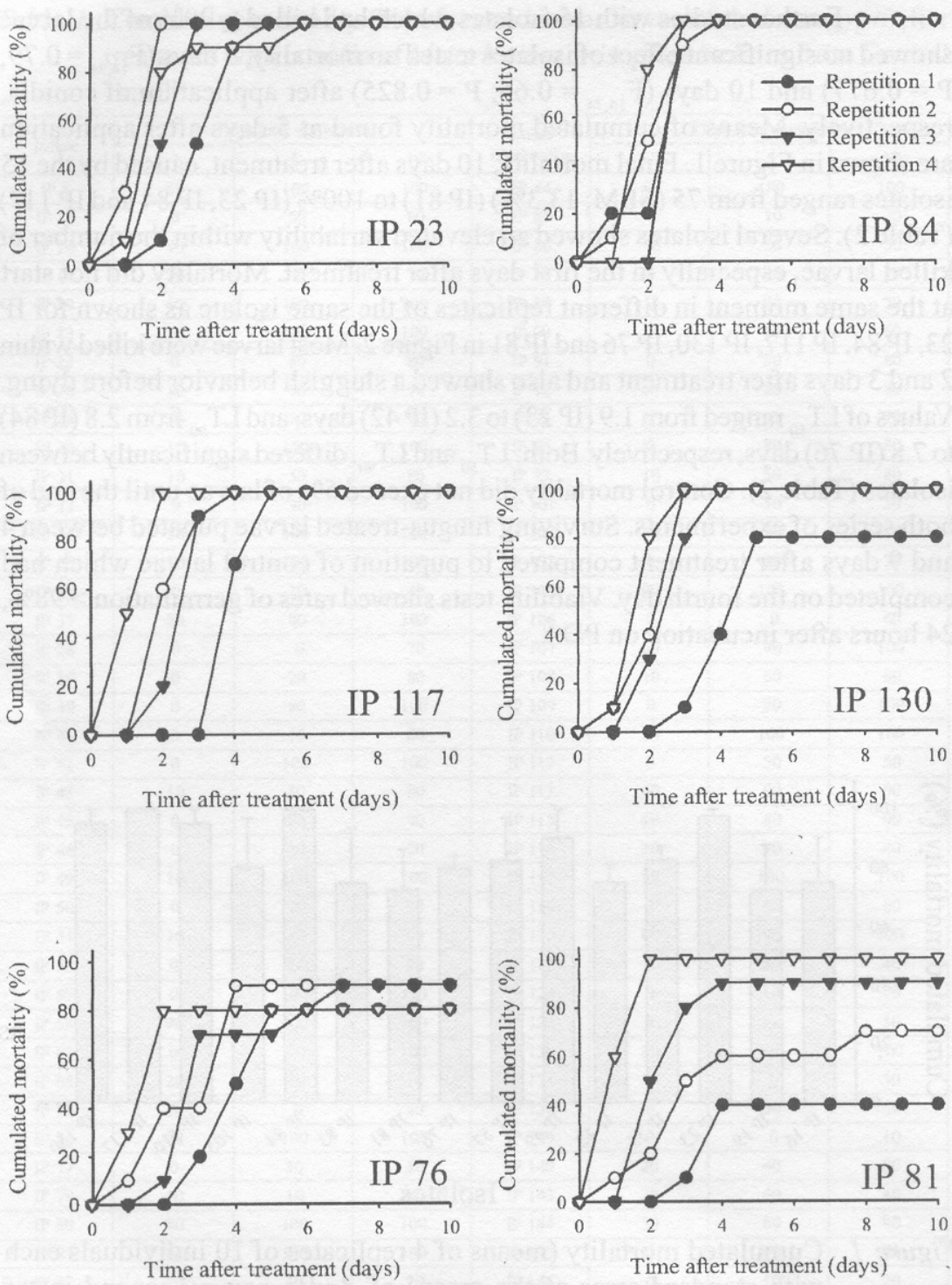

Figure 2. Variability of mortality of *Aedes aegypti* second instar larvae within 4 repetitions after application of *Metarhizium anisopliae* isolates from Central Brazil at 5 x 10^6 conidia/ml

Table 2. Cumulated mortality (%) (SEM: standard error of the mean) and lethal time ($LT_{50/90}$) with respective 95% confidence intervals (95% C.I.) to kill 50 and 90% of second instar larvae of *Aedes aegypti* after treatment with *Metarhizium anisopliae* conidia*

Isolate	Mortality % (means ± SEM) 10 days after treatment	Lethal time (days) to kill 50 and 90% of larvae (95% C.I.)	
		LT_{50}	LT_{90}
IP 16	82.5^{a} (14.4)	2.8^{b} (2.5-3.2)	6.4^{c} (5.4-8.2)
IP 19	82.5^{a} (11.1)	2.9^{b} (2.5-3.3)	7.3^{c} (6.0-9.9)
IP 23	100^{a} (0)	1.9^{a} (1.6-2.1)	3.4^{ab} (3.0-3.9)
IP 35	87.5^{a} (4.8)	2.8^{b} (2.5-3.1)	5.8^{c} (5.0-7.2)
IP 42	80.8^{a} (9.2)	3.2^{b} (2.9-3.6)	7.1^{c} (5.9-9.3)
IP 49	95.0^{a} (5.0)	2.6^{b} (2.3-3.0)	4.8^{bc} (4.3-5.7)
IP 57	85.0^{a} (11.9)	2.6^{b} (2.3-2.9)	5.4^{c} (4.6-6.7)
IP 76	85.0^{a} (2.9)	2.8^{b} (2.4-3.2)	7.8^{c} (6.2-10.1)
IP 81	75.0^{a} (13.3)	2.4^{ab} (2.0-2.9)	7.1^{c} (5.2-11.6)
IP 83	90.0^{a} (7.1)	2.7^{b} (2.3-3.0)	7.1^{c} (5.9-9.2)
IP 84	100^{a} (0)	2.1^{ab} (2.0-2.3)	2.8^{a} (2.6-3.3)
IP 105	85.0^{a} (15.0)	2.6^{b} (2.3-2.9)	5.4^{c} (4.6-6.7)
IP 110	95.0^{a} (5.0)	2.2^{ab} (1.9-2.4)	3.8^{b} (3.4-4.5)
IP 117	100^{a} (0)	2.1^{ab} (1.3-2.6)	3.5^{abc} (2.7-5.0)
IP 130	95.0^{a} (5.0)	2.3^{ab} (2.0-2.6)	4.3^{bc} (3.8-5.2)

*Four replicates of 10 individuals were tested for each isolate at 5 x 10^{6} conidia/ml. Analysis of variance of cumulated mortalities: DF = 14; 59, F = 1.23, P = 0.290. Values within the same column followed by the same letter (*a, b, c*) are not significantly different.

DISCUSSION

M. anisopliae isolated from soil samples in the Brazilian Cerrado were active against larvae of *A. aegypti*. However, initial screening tests showed that activity varied among isolates. Other authors also observed an isolate-dependent virulence of *M. anisopliae* and other hyphomycetic fungi, such as *T. cylindrosporum* (6, 25, 31). In addition to genetically based virulence, activity of *M. anisopliae* and other hyphomycetic fungi in mosquito larvae has been related to susceptibility of mosquito species (24), molting period of the larvae (10), kind, dose and viability of fungal propagules (6, 20, 25), the route of invasion (10, 16, 24), recent host-passage (6), formulation and application techniques (7, 16). In this study, we used unformulated, suspended conidia at a final 5 x 10^{6} conidia/ml with viability > 98% for all isolates, which had been host-passaged prior to the tests. Larvae which died up to 3 days after treatment could be affected after ingestion of conidia or conidia attached to the cuticle. Larval mobility seemed to be inhibited, which may reflect on toxic substances released by partially digested ungerminated conidida in the gut or ungerminated conidia on the cuticle. *M. anisopliae* produces toxic complexes of enzymes (15) or toxins such as destruxins with insecticidal activity (13, 19). The same sluggish

behavior was reported for *A. aegypti* larvae within 48 hours (28) and for *C. quinquefasciatus* larvae within 6 to 24 hours after application of *M. anisopliae* conidia (5, 16). Lacey et al. (16) observed initial stages of germination which were not visible by light microscopy on ultrathin sections of a gut completely packed with *M. anisopliae* conidia of a moribund *C. quinquefasciatus* larva. A rapid kill of larvae within two days after application of conidia was also observed for other hyphomycetic fungi and different mosquito species (4, 21, 22, 23).

Larvae may also have succumbed due to asphyxiation after obstruction of the tip of the respiration siphon by ungerminated conidia. Asphyxiation was reported for *C. quinquefasciatus* larvae which suffocated after contamination of the siphon by floating conidia and development of mycelium in the trachea 48 hours post-treatment, but no contamination of the siphon was observed after application of suspended conidia (16). Larvae in our study may have starved to death due to a massive contamination of the oral brushes with conidia and mechanic interruption of filtration. Differences between initiation and intensity of larval mortality among isolates or replicates of the same isolate were probably related to quantitative external contamination and ingestion of conidia. It cannot be ruled out that larvae which died between 3 and 10 days succumbed to fungal infection after invasion of the larvae via the cuticle or digestive tube.

A bioinsecticide based on fungal propagules or their toxic metabolites which induces rapid and high activity in all larvae is of notable interest. Even though there is a potential of *M. anisopliae* to produce allergic airway disease in mammals, there is actually no evidence of pathogenicity in humans (11, 18). A bioinsecticide based on this fungus is already used for indoor control of cockroaches in the USA. More studies on the potential of *M. anisopliae* and its metabolites under laboratory and under field conditions would be useful in order to develop specific methods of biological control of mosquitoes.

RESUMO

Efeito de isolados de *Metarhizium anisopliae*, obtidos de amostras de solo do Cerrado do Brasil Central, em larvas de *Aedes aegypti* sob condições de laboratório

A atividade larvicida de *Metarhizium anisopliae*, originado de solos do Cerrado do Brasil Central, foi testada em *Aedes aegypti*. A mortalidade de larvas do segundo estádio (L2) variou entre 10 e 100%, 10 dias após a aplicação de 5 x 10^6 conídios/ml de 80 isolados. Em alguns isolados observaram-se paralisia de movimentos e morte acelerada das L2 poucas horas após tratamento. As larvas que sucumbiram 24 horas após tratamento foram encontradas recobertas por um número elevado de conídios não germinados. Uma concentração de conídios

foi observada na ponta do sifão e nas escovas orais dessas larvas, cujo intestino mostrava-se geralmente repleto de conídios não germinados. Em alguns isolados observou-se uma variação do início da mortalidade das L2 entre 24 horas e 3 dias para diferentes repetições. Os valores de TL_{50} variaram de 1,9 (IP 23) até 3,2 (IP 42) dias, e os TL_{90}, de 2,8 (IP 84) até 7,8 (IP 76) dias. Os isolados de *M. anisopliae* induziram mortalidade rápida e alta, o que mostra o seu potencial para controle biológico de *A. aegypti*.

DESCRITORES: *Aedes aegypti*. *Metarhizium anisopliae*. Controle biológico. Cerrado.

ACKNOWLEDGMENTS

To Mark S. Goettel for the critical review of the manuscript, to the National Council of Scientific and Technological Development (CNPq Centro-Oeste) and to the Coordination for the Improvement of Higher Education Personnel (CAPES) for financial support.

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