CHARACTERIZATION AND VIRULENCE OF A BRAZILIAN ISOLATE OF *METARHIZIUM FLAVOVIRIDE* GAMS AND ROZSYPAL (HYPHOMYCETES)

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Abstract *Memoirs of the Entomological Society of Canada* **171**: 313–321 (1997)

A new isolate of *Metarhizium flavoviride* Gams and Rozsypal (Hyphomycetes) (CG 423) found in Northeast Brazil infecting *Schistocerca pallens* (Thunberg) was identified using arbitrarily primed PCR. Cluster analysis of DNA markers revealed a high level of homogeneity (>83% similarity) among the Brazilian (CG 423) and two other *M. flavoviride* isolates from Nigeria (CG 366 = IMI 330189) and Australia (CG 291). However, *M. flavoviride* isolates were very distinct when compared with two isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin (6.4% similarity). Bioassays showed that strain CG 423 is as virulent as other isolates of *M. flavoviride* (CG 291, CG 366), *M. anisopliae* (CG 087), and *Beauveria bassiana* (Balsamo) Vuillemin (CG 425) against the grasshopper *Rhammatocerus schistocercoides* (Rehn) (Orthoptera: Acrididae), an important pest in Central Brazil. However, the Brazilian isolate of *M. flavoviride* (CG 423) is more virulent than the Brazilian isolate of *B. bassiana* (CG 250). Because conidia used in bioassays were formulated in soybean oil containing 5% kerosene, the effect of the kerosene present in the oil formulation was tested. Kerosene (0–10%) did not affect the virulence ($P>0.3$) of *M. flavoviride* against *R. schistocercoides*. The native isolate of *M. flavoviride* (CG 423) is now being developed as a mycoinsecticide against grasshoppers in Brazil.

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Résumé

Un nouvel isolat de *Metarhizium flavoviride* Gams et Rozsypal (Hyphomycètes) (CG 423), virulent contre *Schistocerca pallens* (Thunberg), a été identifié dans le nord-est du Brésil au moyen de la réaction en chaîne des polymérases déclenchée arbitrairement. Une analyse des groupements des marqueurs d'ADN a révélé l'existence d'une forte homogénéité (>83%) entre l'isolat brésilien (CG 423) et deux autres isolats de *M. flavoviride* provenant du Nigeria (CG 366 = IMI 330189) et d'Australie (CG 291). Cependant les isolats de *M. flavoviride* se sont avérés très distincts de deux isolats de *Metarhizium anisopliae* (Metschnikoff) Sorokin (6,4% de similarité). Des expériences ont démontré que le CG 423 est aussi virulent contre le criquet *Rhammatocerus schistocercoides* (Rehn) (Orthoptera: Acrididae), un important ravageur du centre du Brésil, que le CG 366, un agent bioinsecticide en cours de préparation contre les orthoptères d'Afrique. Les conidies utilisées au cours des expériences ont été mises en émulsion dans de l'huile de soja contenant 5% de kérosène. L'effet du kérosène contenu dans la préparation à l'huile a été étudié. Le kérosène (0–10%) n'affecte pas la virulence ($P>0,3$) de *M. flavoviride* contre *R. schistocercoides*. L'isolat indigène de *M. flavoviride* (le CG 423) est en cours de raffinement pour être éventuellement utilisé comme mycoinsecticide contre les orthoptères du Brésil.

[Traduit par la Rédaction]

Introduction

Outbreaks of the grasshopper *Rhammatocerus schistocercoides* (Rehn) and *Schistocerca pallens* (Thunberg) (Orthoptera: Acrididae) have caused severe losses in rice, corn,

TABLE 1. Fungal isolates of *Metarhizium* spp. used in the characterization and bioassay against *Rhammatocerus schistocercoides*

Fungus	Isolate	Original host	Location
M. flavoviride	CG 423	*Schistocerca pallens* (Thunberg) (Orthoptera: Acrididae)	Brazil
	CG 366 (IMI-330189)	*Ornithacris cavroisi* (Finot) (Orthoptera: Acrididae)	Niger
	CG 291 (ARSEF 324)	*Austracnis guttulosa* (Walker) (Orthoptera: Acrididae)	Australia
M. anisopliae var. *anisopliae*	CG 087 (ESALQ PL–43)	Mahanarva posticata (Stål) (Homoptera: Cercopidae)	Brazil
M. anisopliae var. *majus*	CG 320 (ARSEF 297)	*Xyloryctes jamaicensis* (Drury) (Coleoptera: Scarabaidae)	Samoa
B. bassiana	CG 250	Orthoptera	Brazil
	CG 425	*R. schistocercoides* (Orthoptera: Acrididae)	Brazil

sugarcane, and pasture in Brazil (Cosenza et al. 1990). These pests are difficult to control and the application of malathion and fenitrothion is not feasible in Brazil, because of their high cost and possible adverse impact on the environment. The latter has been demonstrated by the effect of fenitrothion and chlorpyriphos on non-target organisms, including entomophagous arthropods and predatory birds that are natural enemies of acridids (Greathead 1992).

Mycoinsecticides based on entomopathogenic fungi such as *Metarhizium flavoviride* Gams and Rozsypal (Lomer et al. 1997; Milner 1997; Magalhães 1994), *M. anisopliae* (Metschnikoff) Sorokin (Lomer et al. 1997), and *Beauveria bassiana* (Balsamo) Vuillemin (Jaronski and Goettel 1997) are under development against grasshoppers in North America and Africa (Goettel, Johnson, and Inglis 1995). These bioinsecticides could supplement or even replace the chemical insecticides currently used in grasshopper control programmes. In Brazil, however, there is little information on native natural enemies (including pathogens) and alternative measures of control (Lecoq and Pierozzi 1994). Therefore, the potential of entomopathogenic fungi as biocontrol agents against *R. schistocercoides* and *S. pallens* populations is being investigated. An isolate of *M. flavoviride*, found in Northeast Brazil infecting *S. pallens*, is being studied for use against this species and *R. schistocercoides*. Recently, the arbitrarily primed polymerase chain reaction (AP-PCR) (Welsh and McClelland 1990), also known as amplified polymorphic DNA (RAPD) markers (Williams et al. 1990), was used to study genetic variability of *Metarhizium* spp. isolates (Bidochka et al. 1994; Fegan et al. 1993; Tigano-Milani et al. 1995). In our study, we report on the molecular taxonomy of a Brazilian isolate of *M. flavoviride* based on AP-PCR analysis, and compare its pathogenicity against *R. schistocercoides* with other isolates of *M. flavoviride*, *M. anisopliae*, and *B. bassiana* from different origins.

Materials and Methods

Fungal Material. Fungal isolates and their origins are listed in Table 1. Isolate CG 366 (IMI 330189) was provided by Chris Prior, IIBC/CAB, Silwood Park, UK, and CG 291 (ARSEF 324) and CG 320 (ARSEF 297) by the United States Department of Agriculture, Agricultural Research Service Collection of Entomopathogenic Fungi, Ithaca, NY. The four

remaining isolates (from Brazil) are deposited in the Collection of Entomopathogenic Fungi at CENARGEN/EMBRAPA, Brasília, DF, Brazil.

Cultures were grown and maintained on Sabouraud dextrose agar medium (40 g dextrose, 10 g mycological peptone, 10 g yeast extract, 10 g agar in 1000 mL of distilled water, pH 5.6) at 25°C, or on complete medium (0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4$, 0.0001% $FeSO_4$, 0.0001% $ZnSO_4$, 0.05% yeast extract, 1% glucose, 0.15% casein hydrolysate, 0.2% peptone, and 1.8% agar) at 27°C.

For the characterization study, the mycelium was obtained from submerged culture in Sabouraud dextrose broth shaking at 150 rpm at 25°C for 3 days. Mycelium was harvested by filtration through filter paper (Whatman #1), and stored at –80°C.

DNA Manipulations. Genomic DNA was extracted from lyophilized mycelium. Approximately 25 mg of lyophilized mycelium was ground to a powder in a 1.5-mL microtube with a sterile toothpick. The powder was then suspended in 500 μL of sterile lysis buffer (100 m*M* Tris-HCl, 100 m*M* NaCl, 50 m*M* EDTA). Fifty microlitres of 10% SDS was added and the tubes shaken gently. After incubation for 10 min at 65°C, 250 μL of cold (4°C) 5 *M* potassium acetate was added, mixed, and the mixture incubated on ice for 20 min. The samples were then centrifuged at 10 000 = *g* at 4°C in a microfuge for 10 min. The supernatant was collected and treated with 5 μL of RNase (10 mg/mL) for 30 min at 37°C. Ten microlitres of proteinase K (10 mg/mL) was added and the mixture incubated for 10 min. The samples were extracted once with 500 μL of phenol/chloroform/isoamyl alcohol (25:24:1; v/v) and once with 500 μL chloroform/isoamyl alcohol (24:1; v/v) at 4°C. Nucleic acids with 0.6 volumes of isopropanol were precipitated, the pellets washed with 70% ethanol, dried, and resuspended in sterile water. The DNA was quantified fluorometrically and verified by agarose gel electrophoresis against a standard DNA of known concentration.

The PCR reactions were performed in a 30-μL volume using a System 9600 cycler (Perkin-Elmer). Two concentrations of each template DNA (10 and 30 ng per reaction) were used to eliminate sporadic amplification products from the data. Elimination of sporadic amplification meant that we used only those products appearing in both concentrations of DNA template. PCR reactions were as described by Tigano-Milani et al. (1995).

Twelve 10-mer primers (Operon Technologies) were used in this study: OPC-03, OPC-06, OPC-11, OPC-14, OPC-16, OPC-18, OPC-19, OPC-20, and OPD-02 were used with AmpliTaq (Perkin-Elmer); OPD-11, OPD-16, and OPD-19 with AmpliTaq Stoffel fragment, according to Sobral and Honeycutt (1993).

The resulting amplified products were resolved by electrophoresis through a 5% acrylamide — 50% urea gel in 1 × Tris-borate EDTA and visualized by autoradiography using x-omat AR film with an intensifying screen at –70°C for 6 h. Urea was included to avoid conformational polymorphisms. Only well resolved products present in both concentrations of the template were scored. The molecular size (in base-pairs) of each band was determined from a single gel regression analysis, and the presence or absence of each amplified fragment of a specific size was considered as an independent character.

Arbitrarily primed PCR characters were analysed using NTSYS-pc v1.8 (Rohlf 1993). A similarity matrix was created using the Jaccard similarity coefficient (Sneath and Sokal 1973), and clustering was done using the unweighted pair group arithmetic mean method (UPGMA) (Sneath and Sokal 1973).

Insects. Third-instar nymphs of *R. schistocercoides* used in this study were collected in Mato Grosso, reared in cages (50 by 50 by 70 cm) at 27°C, and fed leaves of the grass *Andropogon gayanus* Kunth cv. *planaltina*, wheat germ, and textured soybean grains. Insects were chosen randomly without regard to gender.

Bioassay. To compare the pathogenicity of the new Brazilian isolate (CG 423) of *M. flavoviride* against *R. schistocercoides* with other isolates of *M. flavoviride* (CG 366, CG 291),

M. anisopliae (CG 087, CG 320), and *B. bassiana* (CG 250, CG 425), standardized laboratory bioassays were undertaken. Conidia were harvested from the culture plate with the aid of a brush and added to a mixture of soybean oil (95%) + kerosene (5%). Suspensions were adjusted to 10^7 conidia per millilitre. Fungi were topically inoculated (0.5 μL of suspension with 3000 conidia per insect) on the right pleural region of the grasshoppers. After inoculation, insects were housed in cages (17 by 21 by 25 cm; five insects per cage) and fed wheat germ, textured soybean grains, and fresh leaves of *Andropogon* that were renewed every other day. In an initial bioassay we used 20 insects per treatment. In a later bioassay we used 15 insects per treatment. Cages were maintained in a growth chamber at 27°C, in a 12h:12h (light:dark) photoperiod, and 55% relative humidity. In the preliminary bioassay, dead insects were recorded only after 12 days. In the second bioassay, numbers of dead insects were recorded each 12 h and the assay was terminated 12 days after inoculation. To confirm infection, dead insects were maintained in a humid chamber to allow for sporulation of the fungus.

Kerosene was included in the formulation to improve the miscibility of the soybean oil and, consequently, to obtain a more uniform conidial suspension. A possible effect of the kerosene present in the oil formulation on the virulence of *M. flavoviride* (CG 366, our previous standard) against *R. schistocercoides* was tested. Conidia were formulated in soybean oil with different concentrations of kerosene (0, 0.1, 1.0, 5.0, and 10.0%). Final concentration of each suspension was adjusted to 10^7 conidia per millilitre. There were 30 *R. schistocercoides* adults per treatment. The remaining procedure was as described above except that the insects were housed individually in plastic boxes (11 by 11 by 3.5 cm) and mortality was recorded once a day during 10 days.

Statistics. SigmaStat™ and SigmaPlot™ (Jandel Scientific, Corte Madera, CA, USA) were used to calculate statistics and plot data. The median values among the treatment groups in the bioassay were compared using Kruskall-Wallis ANOVA on Ranks and pairwise multiple comparisons were tested using Dunn's method.

Results and Discussion

Characterization. Arbitrarily primed PCR using twelve 10-mer oligonucleotide primers revealed 190 scorable binary characters among isolates of *M. anisopliae* and three isolates of *M. flavoviride*. Amplified products ranged from 2.1 Kb to 172 bp. An example of fingerprinting obtained with two template concentrations, spanning a three-fold concentration range, is shown in Figure 1.

Isolate CG 423, recently found in Brazil, was first classified as *M. anisopliae* var. *anisopliae* by R.A. Humber (ARS/USDA, Ithaca, NY) although the morphological characteristics were intermediate between *M. anisopliae* and *M. flavoviride*. This classification was based on conidial colour and (variable) shapes, general growth and sporulation, and presence of a large proportion of cylindrical phialides. However, based on phenetic analysis of AP-PCR data, this isolate was grouped together with the other two *M. flavoviride* isolates studied (Fig. 2; Table 2). The Brazilian isolate had a pairwise similarity of 95% with the African isolate (CG 366), and 83% with the Australian isolate. The three isolates presented high homogeneity despite their diverse origin (different continents). Bidochka et al. (1994) found little evidence to support the notion that RAPD-PCR patterns were related to either host preference or geographical origin and suggested a clonal nature of *M. flavoviride* based on the genetic homogeneity among its isolates. They also used DNA markers to classify other previously unidentified *Metarhizium* isolates. Our results also confirmed the molecular identification of the isolate CG 291 (ARSEF 324) as being *M. flavoviride* and not *M. anisopliae*, as indicated by the original classification based on morphological characteristics (Bidochka et al. 1994; Bridge et al. 1993; Tigano-Milani et al. 1995).

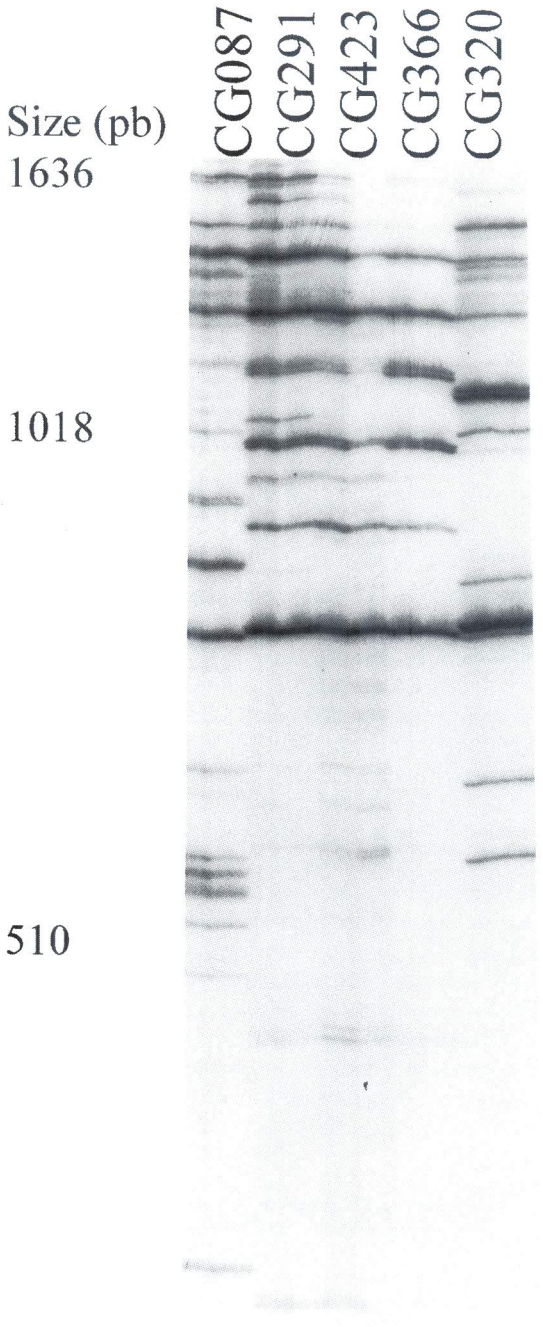

FIG. 1. An example of an autoradiogram of a high-resolution gel showing arbitrarily primed PCR fingerprinting of *Metarhizium* isolates, using the primer OPC-20.

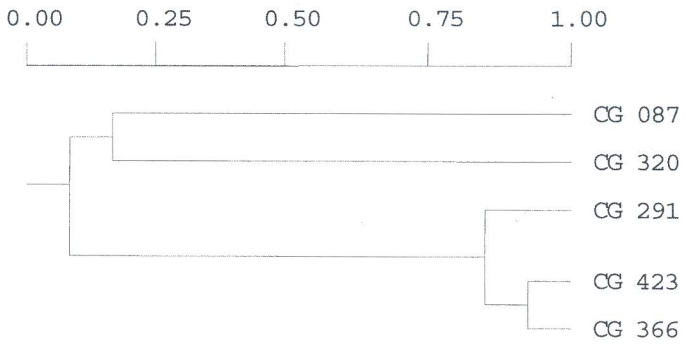

FIG. 2. Dendrogram based on arbitrarily primed PCR data indicating relationship among *Metarhizium* isolates. Similarity matrix based on Jaccard coefficient and tree from similarity matrix by the method of unweighted pair groups, arithmetic mean (UPG-MA).

Metarhizium flavoviride isolates were well separated from *M. anisopliae* isolates (6.4% similarity). *Metarhizium anisopliae* var. *majus* (CG 320) was also very distinct from *M. anisopliae* var. *anisopliae* (CG 087) (10% similarity, Fig. 2).

Bioassay. Similar final mortalities were obtained in both bioassays; however, because daily mortalities were not recorded in the preliminary assay, only results of the second bioassay are presented. Two isolates of *M. flavoviride* (CG 423 and CG 291) caused high mortality (>85%) in *R. schistocercoides* 8 days after inoculation (Fig. 3). Infection was confirmed in all dead insects treated with conidia in both assays. The mean survival time for nymphs was between 108 and 162 h after inoculation (Table 3). It is possible that the lack of statistically significant difference in survival time among CG 423, CG 291, CG 425, CG 366, and CG 087 was related to the high dose (3000 conidia per insect) used in the assays. It is necessary to perform new assays to determine the CL_{50} for each isolate. The low virulence of CG 250 (*B. bassiana*) and CG 320 (*M. anisopliae*) in comparison with all isolates tested ($P<0.05$) suggests a need for the search of new isolates.

Kerosene included in the oil formulation did not affect mortality (<90%, $P<0.3$), survival time (7.7–8.3 days; $P>0.4$), and confirmed infection (>83%, $P<0.7$) caused by *M. flavoviride* (CG 366) on adults of *R. schistocercoides* within the range of concentration tested (0.1–10%) (Fig. 4). Similarly, in another assay, the virulence of *M. flavoviride* (CG 423) against *R. schistocercoides* was not affected by the use of kerosene (0, 5, 10%) to formulate conidia in soybean oil (data not shown). Also, the presence of kerosene in an oat/oil/bait formulation did not affect palatability or efficacy (Goettel, Magalhães, and Gama 1995).

TABLE 2. Similarity coefficients for isolates of *Metarhizium flavoviride* and *M. anisopliae*

Fungus	Isolate	Jaccard similarity coefficient*				
M. anisopliae	CG 087	1.000				
	CG 320	0.101	1.000			
M. flavoviride	CG 291	0.056	0.074	1.000		
	CG 423	0.054	0.079	0.809	1.000	
	CG 366	0.059	0.065	0.853	0.946	1.000

*Based on PCR fragments obtained from twelve 10-mer primers.

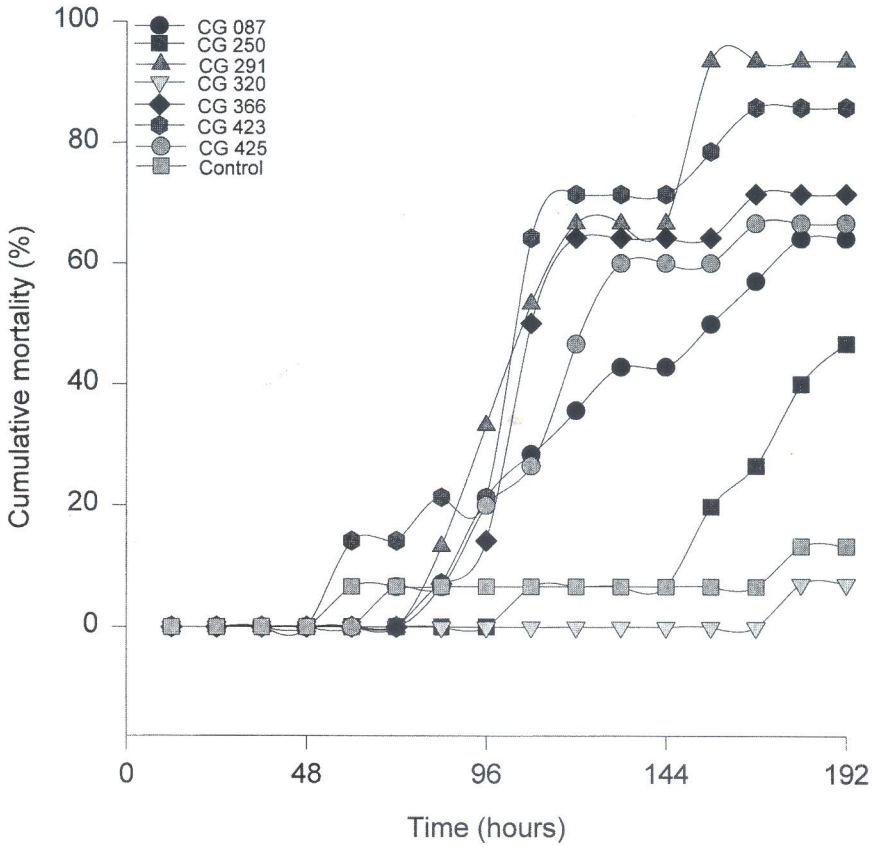

FIG. 3. Mortality of *Rhammatocerus schistocercoides* caused by mycosis of *Metarhizium flavoviride* (CG 291, CG 366, CG 423), *M. anisopliae* (CG 087, CG 320), and *Beauveria bassiana* (CG 250, CG 425) formulated in soybean oil containing 5% kerosene. There were 15 third-instar nymphs per treatment incubated at 27°C in a 12h:12h (light:dark) photoperiod, and 55% relative humidity.

TABLE 3. Survival time of *Rhammatocerus schistocercoides* treated with conidia of *Metarhizium flavoviride*, *M. anisopliae*, and *Beauveria bassiana* formulated in soybean oil containing 5% kerosene

Fungus	Isolate	Survival time (hours)*,†,‡ (mean±SE95)
M. flavoviride	CG 291	117.4±7.3 a
	CG 366	126.5±8.3 ab
	CG 423	108.0±9.2 ab
M. anisopliae	CG 087	150.5±18.5 ab
B. bassiana	CG 425	117.6±9.0 ab
	CG 250	162.8±10.4 b

*Means within columns followed by the same letter are not significantly different by Dunn's method based on Kruskall-Wallis ANOVA on Ranks ($P<0.05$, df = 5).
†There were 15 third-instar nymphs per treatment incubated at 27°C in a 12h:12h (light:dark) photoperiod, and 55% relative humidity.
‡Survival time for CG 320 is not shown because there was only one dead insect in this treatment.

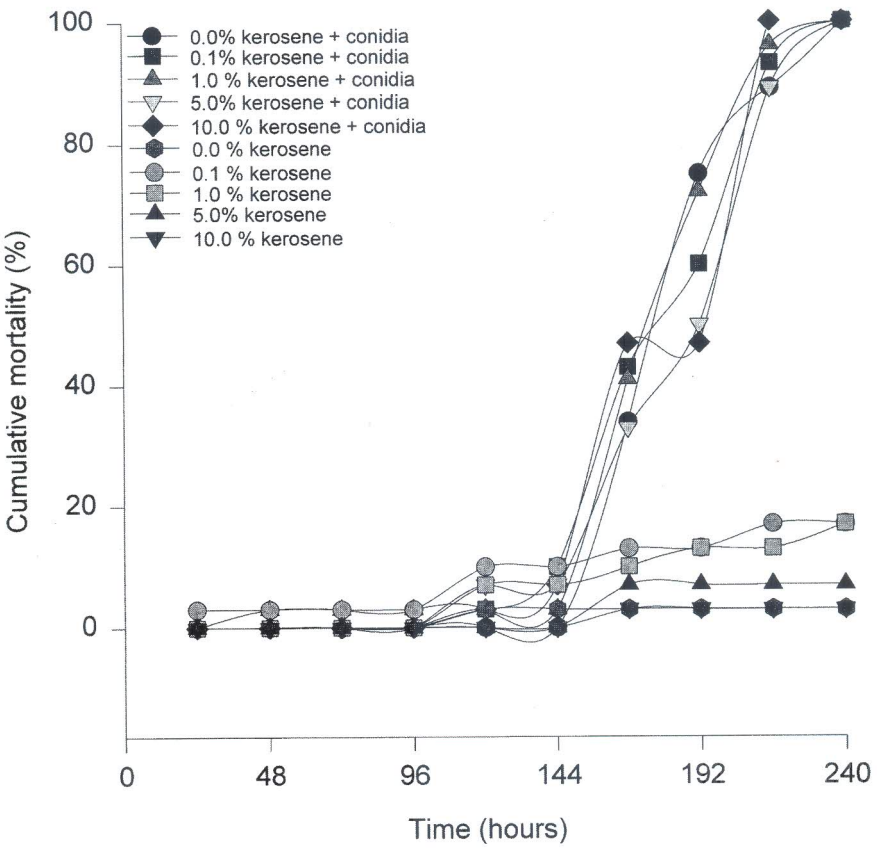

FIG. 4. Mortality of *Rhammatocerus schistocercoides* treated with *Metarhizium flavoviride* (CG 366) formulated in soybean oil containing different concentrations of kerosene (0–10%). There were 30 adults per treatment incubated at 27°C in a 12h:12h (light:dark) photoperiod, and 55% relative humidity.

The use of a native isolate in a microbial control programme is more convenient because it facilitates registration and it poses less environmental risk as compared with exotic isolates. The performance of *M. flavoviride* (CG 423) against *R. schistocercoides* makes it a promising candidate as a mycoinsecticide in Brazil. Because similarity among the isolates of *M. flavoviride* tested in this study is high, other characteristics and different isolates of this fungus are being analysed. Should this genetic homogeneity be confirmed, it will be necessary to introduce a stable marker into the strain selected (CG 423) to evaluate better its efficacy, persistence, and stability in the field. In addition, surveys and screenings (bioassays) of new native isolates of entomopathogenic fungi from grasshoppers and other hosts in Brazil are being intensified.

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