

## NOTAS CIENTÍFICAS

### Characterization of a Peruvian isolate of *Metarhizium anisopliae* var. *acidum*, a pathogen of grasshoppers<sup>(1)</sup>

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Abstract – The objective of this study was to characterize the Peruvian isolate of *Metarhizium anisopliae* var. *acidum*, CG 863, obtained from the grasshopper *Schistocerca interrita*, a crop pest in Peru. The characterization was done by comparing this isolate with two other ones of *M. anisopliae* var. *acidum*, from Brazil and Australia, and with an isolate of *M. anisopliae* var. *anisopliae*. The three *M. anisopliae* var. *acidum* isolates had similar growth profiles in agar plates at 25°C and 37°C, and similar RAPD patterns according to the analysis of three primers. However, regarding these parameters and conidial size, these isolates were very distinct when compared to *M. anisopliae* var. *anisopliae* isolate. Bioassays indicated that the Peruvian isolate is as pathogenic as the Brazilian isolate against nymphs of *Rhammatocerus schistocercoides*.

Index terms: Acrididae, insecta, entomopathogenous fungi, pest control.

### Caracterização de um isolado peruano de *Metarhizium anisopliae* var. *acidum*, um patógeno de gafanhotos

Resumo – O objetivo deste trabalho foi caracterizar o isolado peruano de *Metarhizium anisopliae* var. *acidum*, CG 863, obtido do gafanhoto *Schistocerca interrita*, praga prejudicial a muitas culturas no Peru. A caracterização foi realizada pela comparação com dois outros isolados de *M. anisopliae* var. *acidum*, do Brasil e da Austrália, e com um isolado de *M. anisopliae* var. *anisopliae*. Os três isolados de *M. anisopliae* var. *acidum* mostraram padrões de crescimento semelhantes em placas de ágar a 25°C e 37°C, e semelhantes padrões de RAPD obtidos com a análise de três primers. Entretanto, com relação a estes parâmetros e tamanho de conídios, esses isolados diferiram do isolado de *M. anisopliae* var. *anisopliae*. Bioensaios indicaram que o isolado peruano é tão patogênico a *Rhammatocerus schistocercoides* quanto o isolado brasileiro.

Termos para indexação: Acrididae, inseto, fungo entomopatogênico, combate às pragas.

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Grasshopper outbreaks have occurred in South America for many decades (Beingolea-Guerreiro, 1995; Miranda et al., 1996). In Peru, the migratory grasshoppers *Schistocerca piceifrons piceifrons* and *S. interrita* constitute serious agricultural problems since at least 1901 (Beingolea-Guerreiro, 1995). The entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* has been found in Peru infecting *S. interrita*. This pathogen also occurred in northeastern Brazil infecting *S. pallens* (Moreira et al., 1996) and has been developed as mycopesticide against grasshoppers in Brazil (Magalhães et al., 2000, 2001), Africa (Lomer et al., 1997) and Australia (Milner, 1997). A correct identification and characterization of a particular isolate is very important when developing a pathogen as biocontrol agent.

The objective of this study was to characterize a Peruvian isolate of *M. anisopliae* var. *acridum*, obtained from *S. interrita*, a serious crop pest in Peru.

The fungal isolates used are deposited in the Collection of Entomopathogenic Fungi at Embrapa-Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil (Tigano et al., 2002). The Peruvian isolate of *M. anisopliae* var. *acridum*, codified as CG 863, was collected in Peru (Cajamarca State). The identification and characterization were carried out by comparing this isolate with two other ones of *M. anisopliae* var. *acridum*, from Brazil (CG 423) and Australia (FI 985), and with an isolate of *M. anisopliae* var. *anisopliae* (FI 1029), obtained from another Orthoptera. Cultures were maintained on Sabouraud dextrose agar with 1% yeast extract (SDAY), with a 12-hour photophase at 25°C.

The conidial morphology following growth on SDAY plates was studied using fresh preparations in water with the aid of a microscope. For each isolate, 50 conidia were measured. The rate of growth on SDAY was assessed by inoculating the center of a Petri plate (90 mm diameter) with a filter paper circle (6 mm diameter) embedded in conidial suspension, and by maintaining the plates at two temperatures, 25°C and 37°C. For each temperature and isolate, four plates (replicates) were prepared. The radial growth was measured at seven and 14 days after inoculation.

Random amplified polymorphic DNA (RAPD) analysis was conducted according to Welsh & McClelland (1990) and Williams et al. (1990). Mycelium was obtained from a submerged culture in Sabouraud dextrose broth shaken for three days at 150 rpm, at 25°C. Mycelium was harvested by filtration through filter paper (Whatman No. 1), lyophilized and stored at -80°C. Genomic DNA was extracted using a universal rapid salt method (Aljanabi & Martinez, 1997). The PCR reactions were performed in 30- $\mu$ L volume, with 15 ng of each template, using the PTC-100 programmable thermal controller, and a temperature profile already described (Tigano-Milani et al., 1995). The amplifications were done using the following reaction mix: 1 unit of Taq polymerase, 1x Taq polymerase reaction buffer, 4 mM of MgCl<sub>2</sub>, 200  $\mu$ M of each deoxynucleotides triphosphate, and 1  $\mu$ M of 10-mer primer. Primers were those used by Magalhães et al. (1997) from random primer kit (OPD-92, OPD-011 and OPD-16), and were subjected to electrophoresis in 2% agarose gel dissolved in 0.5x Tris-borato-EDTA (TBE) buffer. After electrophoresis, gels were stained

with ethidium bromide (Sambrook et al., 1989) and photographed under UV light. DNA fingerprints were analyzed visually from the photographs.

The insects used in this study were third and fourth instar nymphs of *R. schistocercoides*, collected in Mato Grosso state (Brazil), reared in cages (50x50x70 cm), maintained at 27°C in laboratory, and fed with sugarcane leaves, wheat germ, and textured soybean grains. The virulence of the Peruvian isolate against insects, chosen randomly, of *R. schistocercoides* was compared to the virulence of the Brazilian isolate by two standardized laboratory bioassays according to Magalhães et al. (1997). Briefly, conidia were harvested from the SDAY culture plate with the aid of a brush and suspended in emulsifiable soybean oil. Suspensions were adjusted to 10<sup>7</sup> conidia/mL and applied topically (3,000 conidia/insect) on the right pleural region of the grasshoppers. After inoculation, insects were held in cages (17x21x25 cm; 10 insects/cage) and fed as described above. Cages were maintained in the laboratory at 25°C to 27°C. Dead insects were recorded each 24 hours and the assays were ended 11 days after inoculation. There were 30 insects per treatment in both bioassays.

The micrometric measurements showed that conidia of the Peruvian isolate (CG 863) are very similar to the conidia of the Brazilian isolate (CG 423) of *M. anisopliae* var. *acridum*. They produce ovoid conidia measuring approximately 5x2.7 µm. However, the conidia of the Australian isolate (FI 985) are almost cylindrical, and significantly larger (P<0.01) than the Brazilian and Peruvian isolates (Table 1). These measurements regarding the three isolates are in agreement with the description given by Driver et al. (2000) for the variety *acridum* of *M. anisopliae*.

The growth of the three *M. anisopliae* var. *acridum* isolates was alike in agar plates at 25°C and 37°C (Table 2). In contrast, *M. anisopliae* var. *anisopliae* (FI 1029) had significantly higher rate of growth (P<0.01) than the *M. anisopliae* var. *acridum* isolates at 25°C, and did not grow at 37°C.

**Table 1.** Conidial length (µm) and width (µm) of *Metarhizium anisopliae* var. *acridum* (CG 863, CG 423, FI 985) and *M. anisopliae* var. *anisopliae* (FI 1029)<sup>(1)</sup>.

Variable	CG 863	CG 423	FI 1029	FI 985
Length	4.94±0.06a	5.04±0.05a	6.88±0.13b	7.57±0.12c
Width	2.73±0.01a	2.76±0.02a	2.82±0.03b	3.04±0.04c

<sup>(1)</sup>Means followed by the same letter in line are not significantly different at 1% probability level by the pairwise multiple comparison procedure (Student-Newman-Keuls method); data are means±standard error of 50 measurements.

**Table 2.** Colony diameter (mm) of *Metarhizium anisopliae* var. *acridum* (FI 985, CG 423, CG 863) and *M. anisopliae* var. *anisopliae* (FI 1029) on solid medium (SDAY), at 25°C and 37°C<sup>(1)</sup>.

Isolate	25°C		37°C	
	7 days	14 days	7 days	14 days
FI 985	17.0±0.0a	36.3±2.5a	11.0±0.0a	17.0±0.5a
CG 423	18.5±1.7a	35.5±0.6a	9.0±0.0b	10.7±0.0b
CG 863	18.0±0.0a	35.3±0.9a	9.0±0.0b	10.0±0.0b
FI 1029	30.0±0.0b	62.5±1.0b	0.0	0.0

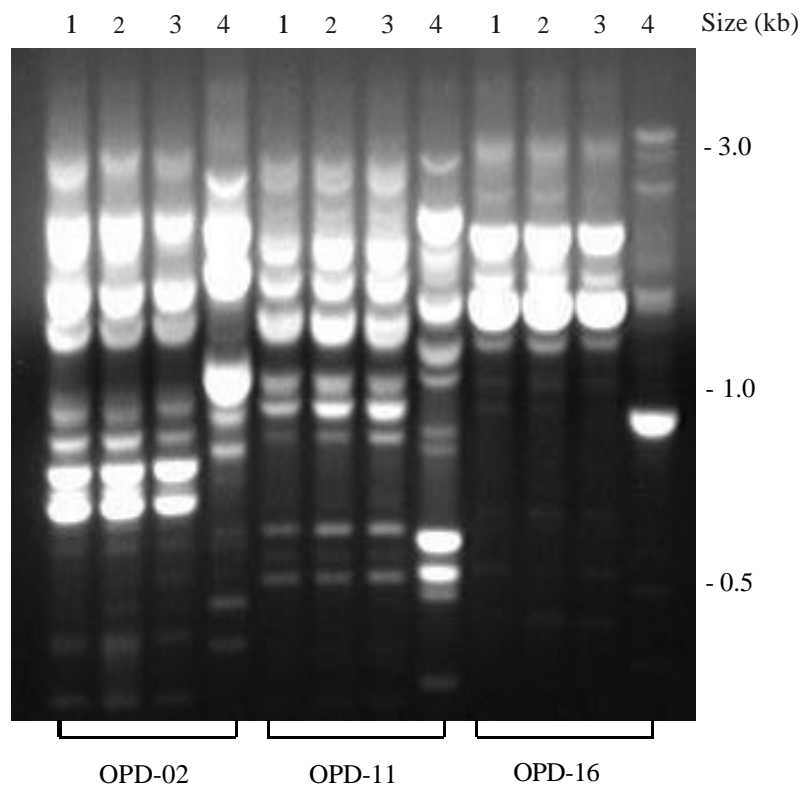
<sup>(1)</sup>Means followed by the same letter in column are not different at 1% probability level by the pairwise multiple comparison procedure (Student-Newman-Keuls method); data are means±standard error of four replicates.

This unusual ability showed by *M. anisopliae* var. *acidum* to grow at 37°C had already been reported (Driver et al., 2000).

The RAPD analysis indicated that the isolates of *M. anisopliae* var. *acidum* have similar patterns which are very distinct from the patterns of *M. anisopliae* var. *anisopliae* (Figure 1). This observation confirms the usefulness of the RAPD technique to analyze polymorphism amongst *Metarhizium* varieties (Magalhães et al., 1997; Driver et al., 2000; Milner et al., 2003).

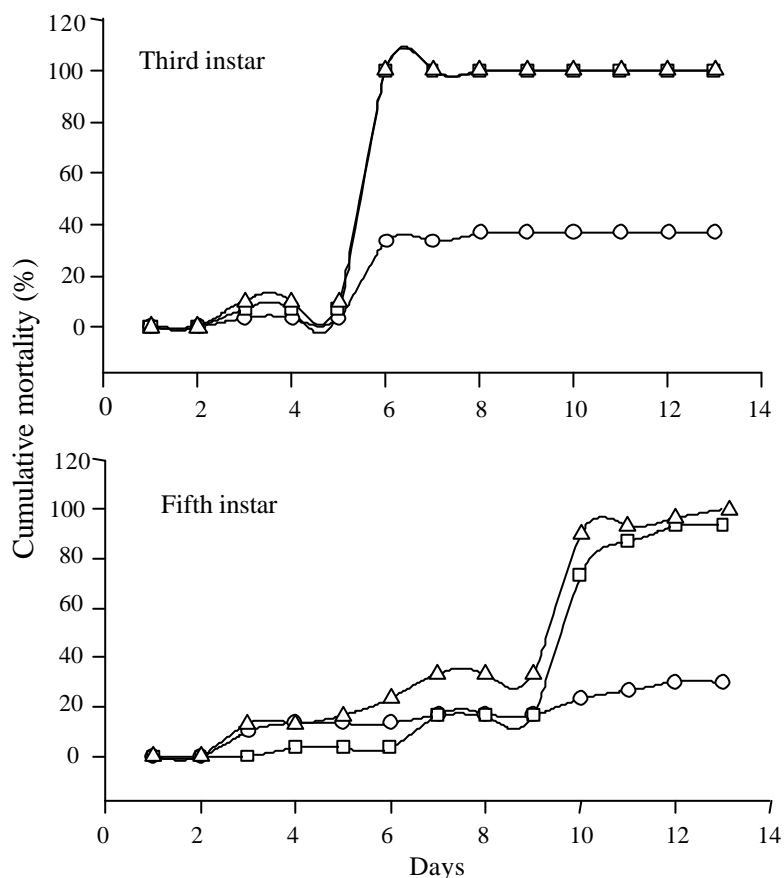
The Brazilian isolate is as virulent to nymphs of *R. schistocercoides* as the Peruvian isolate (Figure 2). In another study, Magalhães et al. (1997) found similar results when the isolate CG 423 was compared to the isolate FI 985. Isolates of *M. anisopliae* var. *acidum* are found mostly within the Acrididae and it is difficult to find natural infections. In contrast, natural infections caused by the variety *anisopliae* are easier to find, but their isolates are not as virulent against grasshoppers as the variety *acidum* (Driver et al., 2000).

In conclusion, the Peruvian and Brazilian isolates of *M. anisopliae* var. *acidum* are very close in terms of growth, conidial morphology and size,



**Figure 1.** Agarose gel showing results from RAPD fingerprint, using the primers OPD-02, OPD-11 and OPD-16 of *Metarhizium anisopliae* isolates. Lanes 1, 2 and 3: *M. anisopliae* var. *acidum*, isolates FI-985, CG 423 and CG 863, respectively; lane 4: *M. anisopliae* var. *anisopliae* isolate FI-1029.

RAPD patterns, and activity against *R. schistocercoides*. This homogeneity among the *M. anisopliae* var. *acridum* isolates has been reported in other studies (Magalhães et al., 1997; Drive et al., 2000; Milner et al., 2003). However, further studies including biology, characterization, and activity of a higher number of isolates from other South America regions are needed. Should these traits be confirmed, it would be reasonable to consider the development of a mycopesticide based on a single isolate to control grasshopper pests in the South America continent. This is particularly attractive if the need to lower the production costs and increase market size for biological control products is considered. An immediate consequence of using a single isolate for the entire region would be a significant reduction in the cost of the registration process, one of the most expensive steps in developing a mycopesticide.



**Figure 2.** Cumulative mortality of *Rhammatocerus schistocercoides* third and fifth instar nymphs treated with the isolate CG 863 (□) and the isolate CG 423 (△) of *Metarhizium anisopliae* var. *acridum*, and control (O).

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