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# DIFFERENTIATION OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM FLAVOVIRIDE* (HYPHOMYCETES)

**Solange Xavier-Santos[1], Bonifácio Magalhães[1*], Elza A. Luna-Alves Lima[2]**

[1]EMBRAPA - Recursos Genéticos e Biotecnologia, Área de Controle Biológico, Brasília, DF, Brasil.
[2] Universidade Federal de Pernambuco, Departamento de Micologia, Recife, PE.

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**SHORT COMMUNICATION**

## ABSTRACT

The differentiation of a Brazilian isolate of *Metarhizium flavoviride* (CG 423), a promising candidate for the biocontrol of grasshoppers, was investigated. Conidia were spread onto solid medium (1% yeast extract, 2.8% agar, 96.2% distilled water), incubated at 28°C and observed during 26 h. Germination initiated as conidia size increased from 5.3 (±0.6) x 3.1 (±0.3) µm (0 h incubation) to 8.1 (±0.2) x 6.1 (±0.2) µm (8 h incubation). Germ tubes started to appear after 10 h incubation showing a high degree of multipolarity. Twenty six hours after inoculation, hyphal differentiation and anastomosis among hyphae from adjacent conidia were recorded. Appressoria were formed only from conidia incubated in liquid medium containing minimum concentration of yeast extract (0.06%; w/v). Appressoria were firmly adhered to the bottom of plastic dishes.

**Key words:** Hyphomycetes, entomopathogenic fungus, germination, appressorium

The fungus *Metarhizium flavoviride* is a promising candidate for the biological control agent of grasshoppers (3, 10) showing encouraging results in Africa (4) and Australia (8). In Brazil, this entomopathogen was originally isolated from *Schistocerca pallens* (Orthoptera: Acrididae) in 1992 (9). Ever since, this pathogen has been studied with the aim of efficiently controlling different grasshopper pests, including *Rhammatocerus schistocercoides* (6) and *S. pallens* (14).

Germination is one of the first steps in the infection process actively affecting pathogenicity or virulence. High virulent strains tend to germinate more quickly than strains with low virulence (7). For many fungi, the pathogenic process is also related to the formation of infection structures or appressoria after germination, which aid the fungus in the invasion of the host cuticle. *Metarhizium anisopliae*, *Beauveria bassiana*, *Aspergillus parasiticus*, *Paecilomyces farinosus, Coelomomyces psorophorae* and *Zoophthrora radicans* are examples of entomopathogenic fungi forming these structures (7, 11).

However, appressorium formation is not a universal prerequisite for successful infection and some fungi are able to penetrate their host cuticle without morphological differentiation. In contrast to other morphological events, appressorium

* Corresponding author. Mailing address: EMBRAPA - Recursos Genéticos e Biotecnologia, Caixa postal 02372, CEP 70849-970, Brasília, DF, Brasil. Fax (061) 340-3624. E-mail: boni@cenargen.embrapa.br

differentiation may not always appear in the life cycle, but be a specific reaction to the environment (12). In this study we investigated the patterns of *in vitro* germination and appressorium formation of *M. flavoviride*.

*Metarhizium flavoviride* (CG 423) used in this study was isolated from *S. pallens* in Rio Grande do Norte state and deposited in the Collection of Entomopathogenic Fungi at Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil.

In order to avoid a possible attenuation process caused by successive transferring and storage, *M. flavoviride* was revigorated by passing it through its host *S. pallens*. After profuse sporulation, conidia were plated on selective medium (2% gross oat Quaker®, 0.1% crystal violet, 0.046% Dodine - monoacetate de N-dodecil-guanidine, 0.05% tetracycline, 2% agar). The fungus was then grown on complete medium (0.4 g $NaHPO_4$, 0.6 g $MgSO_4$, 1 g KCl, 0.7 g $NH_4NO_3$, 10 g glucose, 5 g yeast extract, 15 g agar, 1 liter of distilled water) during 12 days.

Germination was investigated by seeding *M. flavoviride* onto solid medium (1% yeast extract, 2.8% agar) using a conidial suspension of 300 µl ($10^6$ conidia/ml) and incubated at 28°C. Starting immediately after inoculation (0 h), samples were taken at regular intervals of 2 h. At each sample, a culture medium block (± 2 cm²) was prepared for microscopic examination as follows. Samples were fixed for 15 min with formaldehyde (3.7%) and washed with PBS buffer (0.8% NaCl, 0.02% KCl, 0.11% $Na_2HPO_4$, 0.02% $KH_2PO_4$, pH 7.4). To better visualize the fungus development, a cell wall dye Tinopal (Sigma Chemical Co.) was used (1, 5). Preparations were examined and photographs taken using black & white Fuji Neopan-400 film in a fluorescence microscope (Zeiss III R5). Germination and polarity were determined for 100 conidia from each of the three replicates. Conidia was considered to have germinated when germ tubes measured at least their length and those with more than one germ tube were considered multipolars. In addition, conidial dimension (width and length) was recorded before germ tube emergence and growth ratio was determined by measuring germ tube length in a time-course experiment at 2 h intervals, from 10 to 26 h incubation.

To induce appressorium formation, conidia were suspended ($10^5$ conidia/ml) in liquid medium containing yeast extract at two concentrations (0.06% and 1%). The suspension was poured into plastic Petri plates (3.5 cm diameter; 2.5 ml/plate) and incubated at 30°C. A conidial suspension prepared in distilled water was used as control. After incubation during 17 and 30 h, supernatant was removed and the plates were washed with distilled water and stained with Tinopal to examine under a fluorescence microscope the germlings adhered to the bottom of the plates.

SigmaStat™ and SigmaPlot™ (Jandel Scientific, Corte Madera, CA, USA) were used to calculate statistics and plot data. The mean (± SD) is indicated in the figures.

The beginning of the germination process was visualized by the increasing volume of the conidia (Fig. 1). At inoculation, conidial dimensions ranged from 5.3 ± 0.6 µm long by 3.1 ± 0.3 µm wide (at 0 h) to 8.1 ± 0.2 µm by 6.1 ± 0.2 µm (8 h after inoculation). First germ tubes appeared at 10 h incubation and the proportion of germinated conidia increased considerably in the following two hours until the last hour of observation (26 h). By this time, 88.8 ± 4.5 % of conidia had germinated (Fig. 2).

Germ tubes presented uniform growth rate (2.6 ± 0.9 µm $h^{-1}$ ; R = 0.99; y = -20.8 + 2.6 x) until 18 h incubation (Fig. 3). In the following hours, the growth rate increased to15.8 ± 3.8 µm $h^{-1}$ (R = 0.98; y = -328.5 + 18.5 x). At 26 h, germ tubes reached 150 µm and it was possible to see anastomoses between adjacent hyphae. At 14 h incubation, i.e. 4 h after production of the first germ tubes, 45.3% of germlings showed multipolarity (Fig. 4). There was an increasing number of multipolar conidia until 18 h, when 88.2% of the germinated conidia had more than one germ tube, and at 26 h almost all (99.3%) were multipolar. Polarity is related to the accumulation of vesicles containing enzymes responsible for cell wall synthesis in a particular conidial pole (2). Under these conditions, no appressoria were formed at this time. However, when conidia were suspended in the liquid medium with a minimal concentration of yeast extract (0.06%), appressoria were formed against the plastic Petri plate 17 h after inoculation. In contrast, there was no appressorium formation when the yeast extract concentration was 1%. In the absence of yeast extract, germination was very low (< 10%) and no appressoria were formed. The entomophthoralean *Zoophthora radicans* produces appressoria in 1% yeast extract medium but there is no appressorium formation at higher (2%) or lower (0.2 and 0.5%) concentrations (7).

Appressoria formed from swelling hyphal tips (Fig. 5) and were delimited by a septum and firmly adhered to the bottom of the plastic plates. It was not

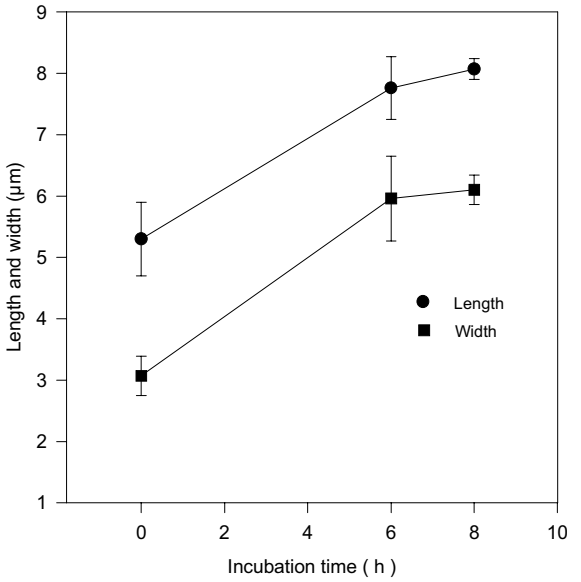


**Figure 1.** Conidial dimensions of *Metarhizium flavoviride* on 1% yeast extract agar at 28ºC.

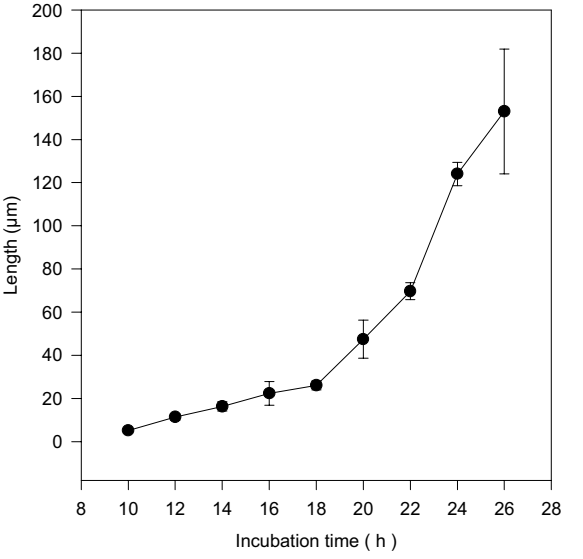


**Figure 3.** Lengtht of *Metarhizium flavoviride* germ tubes on 1% yeast extract agar a 28ºC.

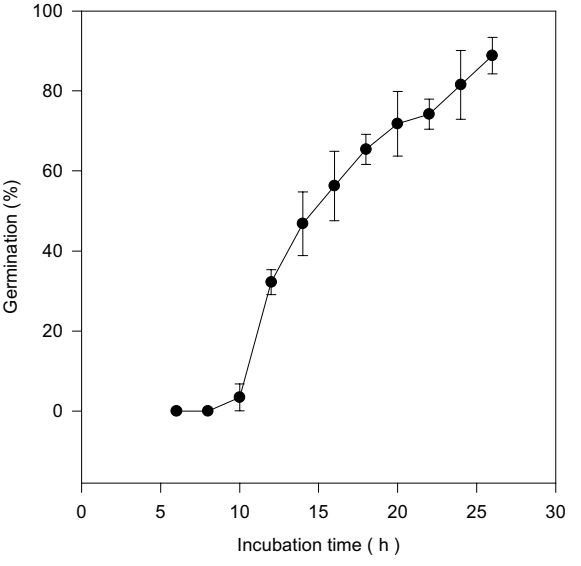


**Figure 2.** Germination of *Metarhizium flavoviride o*n 1% yeast extract agar at 28ºC.

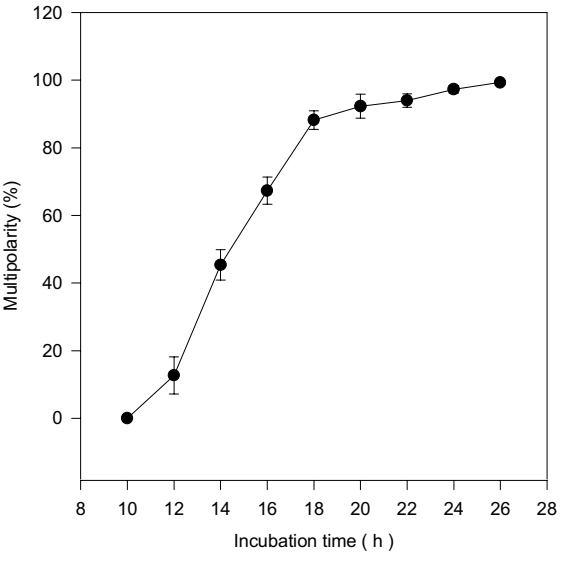


**Figure 4.** Polarity of *Metarhizium flavoviride* germinated conidia on 1% yeast extract agar at 28ºC.

possible to remove these appressoria after several washes. The formation of appressoria after germ tubes contacting a rigid surface was also observed for *M. anisopliae* (12). In this case, the adhesive capability of *M. anisopliae* was attributed to a mucilaginous layer covering the appressorium surface. However, we did not observe such a mucilaginous layer in the development of *M. flavoviride* (CG 423).

In conclusion, germination of *M. flavoviride* starts at 10 h incubation at 28ºC, in a typically multipolar fashion. Differentiation is affected by the environment and nutrition, but appressorium formation is more sensitive to the medium than is conidial germination. A nutrient gradient associated to the germ tube contact with a rigid plastic surface may be the main stimulus triggering appressorium

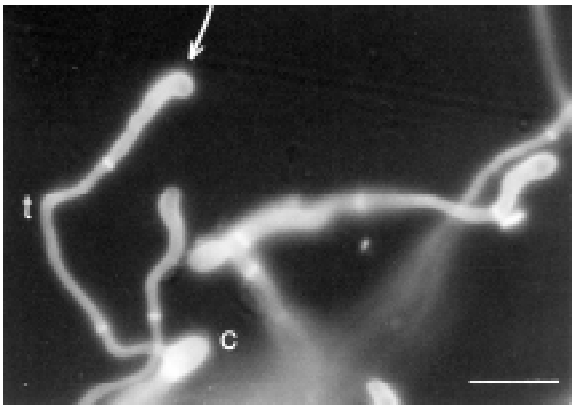


**Figure 5**. Fluorescence micrograph of *Metarhizium flavoviride* (CG 423) grown in yeast extract (0.06%) liquid medium stained with Tinopal (0.5%). Arrow indicates appressoria. t = germ tube, c = conidium (barr = 10µm).

formation. In addition, as observed by St. Leger *et al.* (12), this contact is similar to the natural contact of the germ tube with the rigid insect cuticle. Such a hypothesis is supported by the fact that no appressoria were detected in solid culture medium or in liquid media containing high concentrations of nutrients. A possible explanation for this would be that in the solid medium conidia are onto a soft surface whereas in liquid medium decanted conidia maintain a contact with a rigid surface. *M. flavoviride* formed appressoria on the cuticle of *R. schistocercoides*, but it was able to cause infection without forming appressoria (13). Although our results indicated that appressorium formation by this pathogen is affected by nutritional and environmental factors, further investigation on the actual role of these structures is needed.

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## RESUMO

**Diferenciação do fungo entomopatogênico *Metarhizium flavoviride* (Hyphomycetes)**

A diferenciação de um isolado brasileiro de *Metarhizium flavoviride* (CG 423), candidato a agente de controle biológico de gafanhotos, foi investigada. Conídios semeados em meio de cultura sólido (extrato de levedura 1%, ágar 2,8%, água destilada 96,2%) e incubados a 28°C, foram observados durante 26 horas. Para induzir a formação de apressórios, conídios foram suspensos em meio líquido contendo duas concentrações de extrato de levedura (0,06 e 1%) e transferidos para placas de Petri plásticas (3,5 cm de diâmetro). A germinação teve início com o aumento do tamanho dos conídios de 5,3 ± 0,6 x 3,1 ± 0,3 µm (0 h de incubação) para 8,1 ± 0,2 x 6,1 ± 0,2 µm (8 h de incubação). Os primeiros tubos germinativos começaram a surgir após 10 h de incubação dos conídios, os quais apresentaram acentuada multipolaridade. Vinte e seis horas após a inoculação foi observado o início da diferenciação micelial e formação de anastomoses entre hifas de conídios adjacentes. Apressórios foram formados somente quando conídos foram incubados em meio líquido contendo concentração mínima de nutriente (extrato de levedura 0,0%; peso/volume). Os apressórios formados encontravam-se fortemente aderidos à superfície do fundo plástico da placa de Petri.

**Palavras-chave**: Hyphomycetes, fungo entomopatogênico, germinação, apressório.

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