BIOLOGICAL CONTROL

External Events Related to the Infection Process of *Cornitermes cumulans* (Kollar) (Isoptera: Termitidae) by the Entomopathogenic Fungi *Beauveria bassiana* and *Metarhizium anisopliae*

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Eventos Externos Relacionados ao Processo de Infecção de *Cornitermes cumulans* (Kollar) (Isoptera:Termitidae) Pelos Fungos Entomopatogênicos *Beauveria bassiana* e *Metarhizium anisopliae*

RESUMO - Este estudo teve por objetivos observar o desenvolvimento externo de *Metarhizium anisopliae* e *Beauveria bassiana* em operários e soldados de *Cornitermes cumulans* (Kollar) e comparar duas técnicas de fixação de insetos: secagem até ponto crítico e desidratação com dessecador. Utilizaram-se operários e soldados dos cupins inoculados com *B. bassiana* (447) e *M. anisopliae* (1037). Após a inoculação os insetos foram mantidos a 25 ± 0,5°C. Para as observações amostras das duas castas foram removidas 0, 6, 12, 24, 48, 72, 96, 120, 144 e 168h após a inoculação. A germinação dos conídios de *M. anisopliae* e *B. bassiana* ocorreu em várias regiões do corpo dos insetos principalmente entre 6h e 12h após a inoculação e a penetração ocorreu após 12h e 24h. Vários pontos de penetração originaram-se de um único tubo germinativo. A colonização do hospedeiro pelos dois fungos ocorreu entre 24h e 72h sendo que a maioria dos insetos (>80%) morreu entre 48h e 72h após a inoculação para *B. bassiana*, e 72h e 96h, para *M. anisopliae*. A conidiogênese iniciou-se entre 72h e 96h para *B. bassiana* e entre 96h e 120h para *M. anisopliae.* A conidiogênese para *B. bassiana* iniciou-se mais cedo provavelmente por ter este fungo/isolado maior velocidade de colonização do inseto. Este fator e a existência de vários pontos de penetração, para um mesmo tubo germinativo, podem explicar a maior virulência dos isolados. A secagem até ponto crítico foi a técnica de fixação que melhor preservou as estruturas do patógeno e dos insetos.

PALAVRAS-CHAVE: Controle biológico, controle microbiano, patógeno de insetos, cupim

ABSTRACT - The objectives of this study were to observe the external development of *Metarhizium anisopliae* and *Beauveria bassiana* on workers and soldiers of *Cornitermes cumulans* (Kollar) and to establish comparisons between the insect fixation techniques known as critical point and desiccator. Termite workers and soldiers inoculated with *B. bassiana* (447) and *M. anisopliae* (1037) were utilized. After inoculation, the insects were left at 25±0.5°C. In order to make observations, samples from both castes were removed at 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168h after inoculation. The external development of *M. anisopliae* and *B. bassiana* conidia on the termite *C. cumulans* showed that *M. anisopliae* and *B. bassiana* conidial germination occurred on several regions of the termites body mainly between 6h and 12h and penetration mainly between 12h and 24h after fungal application. Several penetration points were observed originating from the same germ tube. Colonization of the host by *M. anisopliae* occurred between 24h and 72h, and most insects died between 72h and 96h. Conidiogenesis began between 96h and 120h with the peak between 144h and 166h for *M. anisopliae* and between 120h and 144h for *B. bassiana***.** Thus, only conidiogenesis for *B. bassiana* started earlier, probably because this fungus/isolate shows a higher insect colonization speed. This factor, in addition to multiple penetration points for the same germination tube, could explain the higher virulence of the isolates. The critical point fixation technique provided the best preservation of structures in both the pathogen and the insect.

KEYWORDS: Biological control, microbial control, insect pathogen, termite

The life cycle of insect mycopathogens involve the dissemination, adhesion, germination, penetration, invasion and colonization in the hemocoel, tissues, and organs. Conidiogenesis normally occurs after the external growth of the fungus. During fungal growth the production of toxins may occur in the insects body. The timing of each of these stages is variable according to fungal species host and environmental conditions (Alves 1998). Moreover, the time from infection to insect death varies with the dose of applied conidia and the virulence of isolates.

For studies of the cycle of entomopathogenic fungi on insects using scanning electron microscope (SEM), the technique of choice has included glutaraldehyde fixation followed by dehydration in ethanol series, and critical-point drying (Champlin *et al*. 1981, Lecuona *et al.* 1991). Fixation using OsO_4 vapor has also been used (Boucias & Pendland 1982, Gunnarsson 1988). Bidochka & Khachatourians (1992) air-dried specimens for 24h after the fixation and dehydration, in order to study the growth of *Beauveria bassiana* (Balsamo) Vuill. on the cuticle of the acridid *Melanoplus sanguinipes* (Fabricius). Fixation of the specimen by immersion can modify the structures of the pathogen. To avoid these problems with insects treated with *Beauveria* sp., Quattlebaum & Carner (1980) used fixation with OsO_4 for 96h, followed by air-drying for three to four days. Using this technique, the authors obtained minimal distortion of fungal structures. For quantitative studies in which the numbers of fungal structures in different regions of the insect body are important, fixation with OsO_4 vapor for 48h to 96h is recommended to prevent a possible loss of structures. However, when the objective is simply to detect structures with a few or no deformations, other techniques may be used. For insects with thin cuticle such as the mount termite *Cornitermes cumulans* (Kolar), fixation and critical point drying preserve both fungal and insect structures, making detailed observations possible.

Dissemination, penetration, invasion and replication of entomopathogenic fungi in termites have not been studied in details. Whereas entomopathogenic fungi usually take four to five days to kill insect hosts (Alves 1998), termites are usually killed in shorter period of time (Altson 1947, Yendol & Paschke 1965, Bao & Yendol 1971). However, Hanel (1981) observed that mortality of *Nasutitermes exitiosus* (Hill) due to *Metarhizium anisopliae* infection occurred between 8 to 10 days after inoculation. For *M. anisopliae*, the germination and penetration occurred between 24h and 48h. Conidiogenesis occurred in four and five days, respectively for *M. anisopliae* and *B. bassiana.* Studying the cycle of *M. anisopliae* in *N. exitiosus*, Hanel (1982) observed that the fungus penetrated the cuticle of the insect with the occasional formation of one or more apressoria per conidium. The penetration event occurred between 18h and 66h on any region of the insect body, and hyphal bodies were formed between cuticle layers.

The main objective of this study was to observe and compare the external development of the entomopathogenic fungi *M. anisopliae* and *B. bassiana* when inoculated on workers and soldiers of the mound-building termite *C. cumulans*. In addition to providing basic information on the process of external development, these observations allow to identify different infection strategies and developmental velocities that could, in part, explain the higher or smaller virulence found in the isolates. Another objective was to compare two specimen fixation techniques for observation under the scanning electron microscope.

Material and Methods

The *C. cumulans* workers and soldiers used in the experiments were field collected from colonies at the Piracicaba region, São Paulo State, Brazil. The fungal isolates used in the bioassays were B. *bassiana* 447 and *M. anisopliae* 1037 from the entomopathogen collection maintained by the Laboratory of Insect Pathology at the "Escola Superior de Agronomia Luiz de Queiroz" ESALQ - Universidade de São Paulo, Brazil. All the SEM observations were performed with a electron microscope Zeiss, model DMS 940.

Two techniques for fixing the specimens for observation in SEM were tested. In the first technique (desiccator), insects were killed with ether vapor, fixed in OsO_4 vapor for a minimum of 48h, and dehydrated in a desiccator for a minimum of 72h. Fixed insects were coated with gold using a sputter coater and observed under SEM. For the second technique (critical point drying), insects were killed with ether vapor, fixed in 4% glutaraldehyde solution in cacodylate buffer (0.2 M, pH 7.2) for 6h, followed by immersion in 1% OsO4 in cacodylate buffer (0.1 M, pH 7.2) for 1h. Insects were dehydrated in acetone series (30, 50, 70, 90 and three times 100%, with 10 min. per step). Specimens were then critical-point dried under CO_2 before being gold coated and observed under SEM as described above. For each technique, one stub with two workers and two soldiers was prepared for each sampling time as described below, for a total of 20 insects for each caste. Insects were separated in two groups, each with 120 soldiers and 120 workers kept in dishes (100 x 10 mm). Insects were treated (sprayed) in the dishes with 0.5 ml of conidial suspension (1×10^8 conidia ml^{-1}), divided into groups of 10 workers and 10 soldiers and transferred to containers to be used in the bioassays. These containers (4.5 cm diameter; 6.5 cm height) contained a portion of the carton (soft part) from termite nests and a moist filter paper fixed to the lid. Termites were kept in an incubator at 25 ± 0.5°C in the dark. Samples of the two castes were removed 0, 6, 12, 24, 48, 72, 96, 120, 144 and 168h after inoculation.

Results and Discussion

The critical-point drying technique was chosen because it preserved both the insect and fungal features better than the desiccator technique.

***M. anisopliae* Developmental Cycle.** The attachment process for *M. anisopliae* has occurred between application and 6h. Conidial germination took place 6h after application (Fig. 1a) and penetration occurred 12h, without (Fig. 1b) or with (Fig. 1c) the formation of appressorium. Similarly Hanel (1982) observed an average penetration time of 48h for *M. anisopliae* in *N. exitiosus*.

The possible penetration of *M. anisopliae* was observed

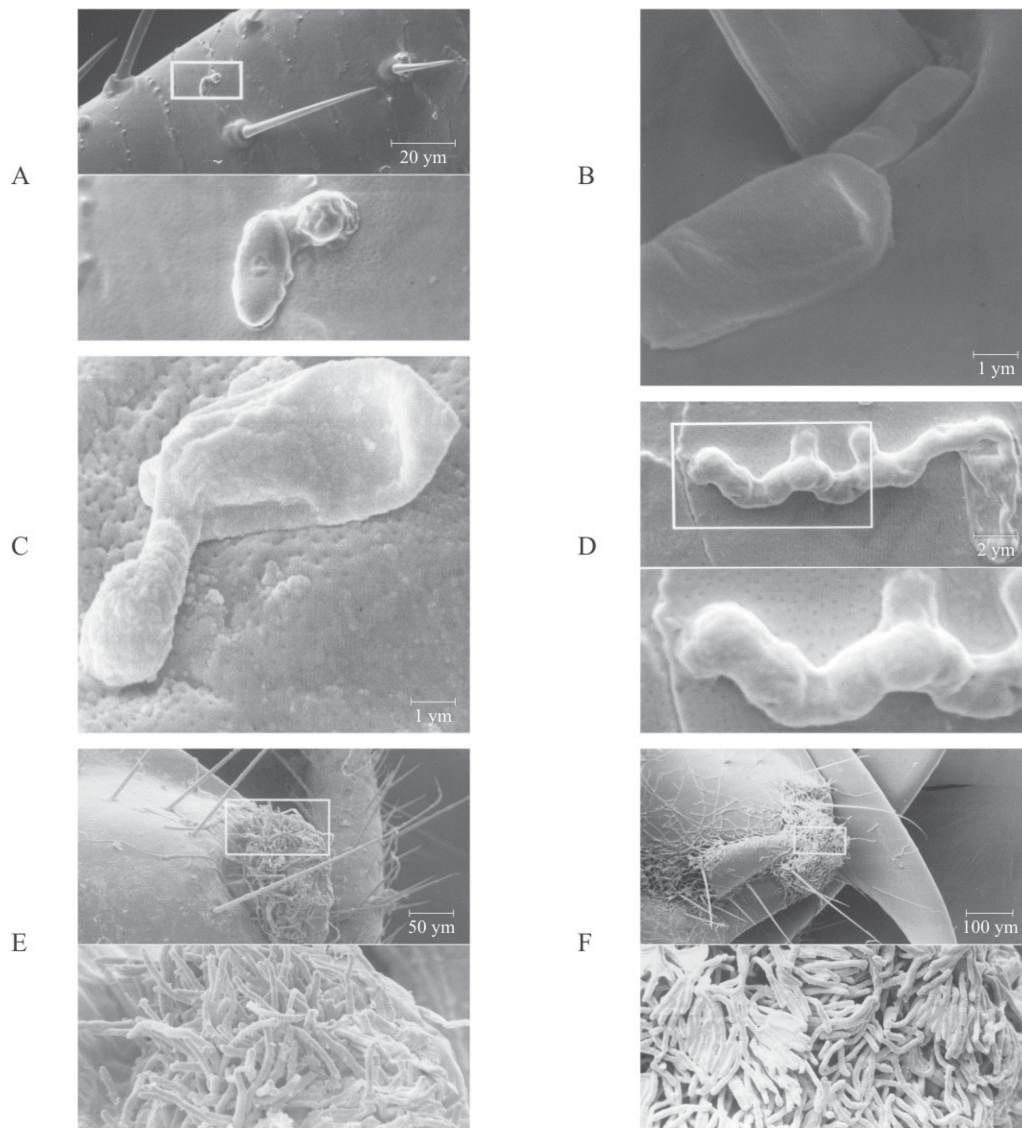

Figure 1. SEM micrographs of *M. anisopliae* on *C. cumulans*: A) Germinating conidia - 12h (500 x 8); B) Possible penetration at base of setae - 24h (10,000 x); D) Conidium germinating with appressorium formation - 72h (10,000 x); D) Conidium germinating with formation of different penetration points - 72h (1,000 x 8); E) Growth of mycelium from the tip of soldier labium - 96h (200 x 4); F) Initial conidiogenisis - 120h (100 x 8).

on the different regions of the termite body, but mainly on the leg segments and on the head. Some germ tubes penetrated at the base of setae (Fig. 1b). Penetration at these sites is probably faster because the cuticle is thinner or softer than in other body areas, so probably lower production of exoenzymes is needed for degradation of the cuticle. Penetration was confirmed by observations in the transmission electron microscope. However, SEM could give an idea of what is happening, in a faster and easier way, when the germination processes and the mortality of the insect by the fungi were observed and confirmed.

Occasionally a halo was apparent around both *B. bassiana* (Fig. 2e) and *M. anisopliae* conidia and germ tubes. This halo probably is associated with the production of fungal exoenzymes during the penetration process. Chimoelastases, such as the enzyme Pr1 produced by *M. anisopliae,* have been observed previously degrading the insect cuticle during the penetration by fungi (Roberts & Krasnoff 1998). According to these authors, at the formation of the appressorium, the synthesis of Pr1 represents 78% of the protein production by *M. anisopliae.* Pr1 is a key factor in the virulence of this fungus. The formation of several penetration points originating from the same germ tube (Figs. 1d and 2c) was observed only occasionally. Multiple penetrations may lead to a more extensive hyphal plate growth between the cuticle layers as observed with *M. anisopliae* in *N. exitiosus* (Hanel 1982) and *B. bassiana* in *Reticulitermes flavipes* (Kollar) (Boucias *et al*. 1996).

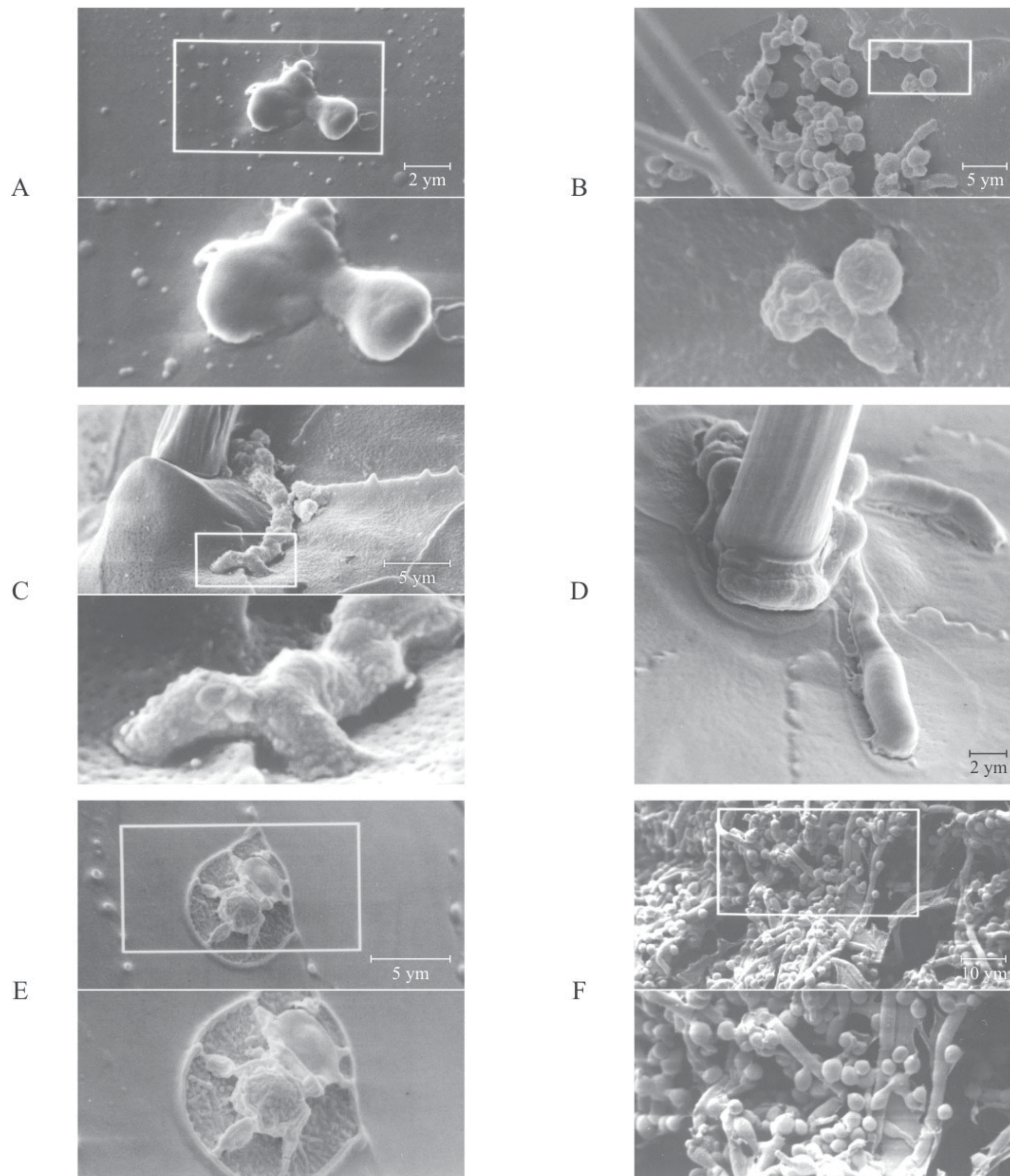

Figure 2. SEM micrographs of *B. bassiana* on *C. cumulans*: A) Germinating conidia - 12h (5,000 x 2); B) Conidia germinating and possible penetration - 12h (2,000 x 4); C) Possible cuticle penetration at two points after bifurcation of the germ tube - 72h (3,000 x 4); D) Possible penetration at the base of a setae – 24h (4,300); E) Germinating conidia – 24h (3,000 x 2); F) Beginning of conidiogenesis - 96h (1,000 x 2).

During the period between 24h and 72h after inoculation, *M. anisopliae* probably colonized the insect. Most insects (ca. 80%) died between 72h and 96h after inoculation. During this period initial mycelium grew out through the insect. Initially this occurred on softer parts of the body as the intersegmental regions (Fig. 1e) or from natural openings such as the extremity of the fontanelle (Fig. 1f). The membranous region at the tip of the labium of the soldiers was the area where the initial external mycelial growth took place (Fig. 1e).

Conidiogenesis was initiated between 96h and 120h after inoculation, but the intensity increased between 120h and 144h and reached the peak between 144h and 166h after inoculation. Higher conidial production occurred on the heavily sclerotiszed regions of the head and the thorax. The head was always the body region with the most intensive mycelial growth and consequently higher conidial production

B. bassiana **Developmental Cycle.** Development of *B. bassiana* on *C. cumulans* was similar to that observed *with M. anisopliae*. The conidial attachment occurred until 6h after application and germination started between 6h and 12h post-

innoculation (Figs. 2a and b). Attached conidia were observed mainly on the head, thorax and legs. Penetration occurred between 12h and 24h after inoculation (Figs. 2b and c).

Structures similar to an appressorium (Figs. 2a and b) appeared between 6h and 12h after inoculation. These structures may be associated with the penetration in the host body, occur early in the developmental cycle of *B. bassiana* isolate 447 and may be the reason for the high virulence of this isolate against the termite species. Although some fungal penetration into the host cuticle was already visible between 6h and 12h after inoculation, the number of germinating conidia was still very low (±10%). On *R. flavipes*, *B. bassiana* germ tubes and penetration structures formed between 12h and 24h after inoculation (Boucias *et al*. 1996). At least some conidia of *B. bassiana* 447 seemed to produce germ tubes that penetrated *C. cumulans* 6h to 36h earlier than the above observations with other termite species. Although high germination rates have been recognized as important determinants of virulence and pathology (Pekrul & Grula 1979, Hassan *et al.* 1989, Yokoyama *et al.* 1993), early penetration may also be associated with these traits. The production of these penetration structures early in the fungal developmental cycle on insect hosts may be an important trait for selection of isolates for use in microbial control programs.

With some conidia, lateral outgrowths were observed branching from the main penetration with characteristics of an appressorium (Fig. 2d). These structures may be associated with the penetration process or the attachment of the conidia to the insect cuticle.

Colonization of the host by *B. basssiana* 447 occurred between 24h and 72h, and the insects died between 48h and 72h after inoculation, faster than *M. anisopliae*. As it is typical in *B. bassiana*-infected insects with a clear cuticle, most cadavers had a pink coloration in the head capsule and the legs before mycelium exited the insect body. These regions had the most extensive external mycelial growth after insect death. Growth of *B. bassiana* out of the cadavers and the beginning of conidiogenesis occurred between 72h and 96h after inoculation (Fig. 2f). As observed with *M. anisopliae*, external growth initiated on the intersegmental regions, spreading over the entire host but especially on the head, the thorax and the legs. With conidiogenesis occurring soon after the mycelium escape from the insect host, *B. bassiana* 447 had faster conidial formation than *M. anisopliae* 1037. Peak sporulation of *B. bassiana* 447 occurred between 120h and 144h after inoculation.

The fungus *B. bassiana* (447) colonized the insects more rapidly than *M. anisopliae*, since they killed the insects and began conidiogenesis more prematurely. This characteristic could be responsible for the greater virulence of the fungus/isolate. The other developmental stages (germination and penetration) did not show differences between the two pathogens. Several points of penetration for the same germination tube were also verified. This observation is not cited in the literature, but it could also be associated with the greater virulence of the fungus/isolate. With respect to the fixation techniques, the critical point technique was more adequate for the preservation of fungal and insect structures.

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