Histopathological events and detection of *Metarhizium anisopliae* using specific primers in infected immature stages of the fruit fly *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae)

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Abstract

The fungus *Metarhizium anisopliae* is used on a large scale in Brazil as a microbial control agent against the sugar cane spittlebugs, *Mahanarva posticata* and *M. fimbriolata* (Hemiptera., Cercopidae). We applied strain E9 of *M. anisopliae* in a bioassay on soil, with field doses of conidia to determine if it can cause infection, disease and mortality in immature stages of *Anastrepha fraterculus*, the South American fruit fly. All the events were studied histologically and at the molecular level during the disease cycle, using a novel histological technique, light green staining, associated with light microscopy, and by PCR, using a specific DNA primer developed for *M. anisopliae* capable to identify Brazilian strains like E9. The entire infection cycle, which starts by conidial adhesion to the cuticle of the host, followed by germination with or without the formation of an appressorium, penetration through the cuticle and colonisation, with development of a dimorphic phase, hyphal bodies in the hemocoel, and death of the host, lasted 96 hours under the bioassay conditions, similar to what occurs under field conditions. During the disease cycle, the propagules of the entomopathogenic fungus were detected by identifying DNA with the specific primer ITSMet: 5' TCTGAATTTTTTATAAGTAT 3' with ITS4 (5' TCCTCCGCTTATTGATATGC 3') as a reverse primer. This simple methodology permits in situ studies of the infective process, contributing to our understanding of the host-pathogen relationship and allowing monitoring of the efficacy and survival of this entomopathogenic fungus in large-scale applications in the field. It also facilitates monitoring the environmental impact of *M. anisopliae* on non-target insects.

Keywords: Metarhizium anastrepha, fruit fly control, histology, PCR analysis.

Eventos histológicos e detecção de *Metarhizium anisopliae* usando primers específicos em estágios imaturos da mosca das frutas *Anastrepha fraterculus* (Wiedemann, 1830) (Diptera: Tephritidae)

Resumo

O fungo *Metarhizium anisopliae* é usado em larga escala no Brasil como um agente de controle microbiológico contra a cigarrinha da cana-de-açúcar, *Mahanarva posticata* e *M. fimbriolata* (Hemiptera, Cercopidae). Aplicou-se a linhagem E9 de *M. anisopliae* em bioensaio no solo, nas dosagens de campo, para se determinar se este poderia causar infecção, doença e morte a estágios imaturos de *Anastrepha fraterculus,* a mosca sul-americana das frutas. Todos os eventos foram estudados histologicamente e ao nível molecular durante o ciclo da doença, usando-se uma nova técnica de coloração, o corante *light Green*, associado com a microscopia de luz, e a técnica do PCR, usando-se *primers* específicos desenvolvidos para *M. anisopliae* linhagens brasileiras, entre elas a E9. O ciclo infectivo, que se inicia pela adesão do conídio sobre a cutícula do hospedeiro, seguido da germinação, com ou sem a formação de apressório, penetração através da cutícula, colonização, com desenvolvimento da fase dimórfica, corpos hifais na hemocela com a morte do hospedeiro, dura 96 horas, nas condições do bioensaio, similar às condições de campo. Durante o ciclo da doença, propágulos do fungo entomopatogênico foram detectados pela identificação do DNA com *primer* específico ITSMet:

5’ TCTGAATTTTTTATAAGTAT 3’ com o ITS4 (5’ TCCTCCGCTTATTGATATGC 3’) como *primer* reverso. Esta metodologia simples permite o estudo in situ do processo infectivo, contribuindo com o entendimento da relação entre patógeno hospedeiro e permitindo o monitoramento da eficácia e sobrevivência deste fungo entomopatogênico em aplicações em larga escala no campo. Esta também permite e facilita o monitoramento do impacto de *M. anisopliae* sobre insetos não alvos.

Palavras-chave: Metarhizium anisopliae, controle de moscas das frutas, histologia, análise de PCR.

1. Introduction

Anastrepha fraterculus, the South American fruit fly, belongs to a group of pests of worldwide economic importance for fruit production. In Brazil, fruit flies of the genus *Anastrepha*, especially *A. fraterculus* and *A. obliqua*, have the widest geographic distribution (Morgante, 1991). Along with *A. sororcula* and *A. grandis*, these flies are classified as quarantine species by protection agencies in various countries like: Venezuela, Argentina, Peru, Jamaica, Mexico, Colombia and also additional species of this genus have been reported as important pest species (Nascimento et al., 1993).

Chemical products have been used for fruit fly control, although there have been efforts to develop an integrated pest management programme using biological control agents (Destéfano et al, 2005); control has also been attempted with bioregulators, such as gibberellic acid (Greany et al., 1991). Among the entomopathogens, species of two other fungal genera, *Paecilomyces fumosoroseus* and *Beauveria bassiana* (Carneiro and Salles, 1994), have been tried against *A. fraterculus*. Garcia et al. (1984, 1985) examined the action of *M. anisopliae* on larvae, pupae and adults of *C. capitata*, and Destéfano et al. (2005) tested this same fungus on *A. fraterculus*. The entomopathogenic mitosporic fungus *Metarhizium anisopliae* has been used in Brazil on a large scale as a microbial control agent against *Mahanarva posticata* and *M. fimbriolata* the sugarcane spittlebugs; more than a million hectares of sugar cane and improved pastures are treated annually (Leite et al., 2003). This fungus has also been used in other countries, including Colombia, Australia, China and the United States of America, in commercial applications to control various different target insects. *Metarhizium anisopliae* has also shown to have good potential in bioassays against *Oncometopia facialis* (Hemiptera: Cicadellidae) (Pria Júnior et al., 2008). Because of the great potential for this fungus as a biocontrol agent, we decided to study the infection process and the disease cycle with histological methods. We also used DNA identification to detect this entomopathogen internally and within the cuticle of the host. The disease cycle was studied in a bioassay on soil, using field dosages of *M. anisopliae* conidia that normally are employed when it is used as a microbial control agent. The entire infection cycle, from adhesion of the conidia to the cuticle, penetration, colonisation and exteriorisation during the disease cycle, was histologically studied with a novel staining preparation and light microscopy, observing the development of the entomopathogen structures. The fungus was also detected in PCR analyses using the DNA extracted from the host at each phase during the infective process; a specific DNA primer ITSMet: 5’ TCTGAATTTTTTATAAGTAT 3’ with ITS4 (5’ TCCTCCGCTTATTGATATGC 3’) as a reverse primer (Destéfano et al., 2004) was employed. Our objectives were to improve our understanding of the host/pathogen relationship to help direct strain selection, for evaluating the efficacy of this fungus in field applications, and for monitoring the environmental impact of *M. anisopliae* on non-target insects.

2. Material and Methods

2.1. Preparation of infective units

Conidia (the infective units) were obtained from a culture of strain E9 of *M. anisopliae* isolated from *Deois flavopicta* (Hemiptera, Cercopidae) grown on rice medium (100g rice/80 ml H_2O autoclaved for 20 minutes at one atmosphere) in Erlenmeyer flasks and harvested 10 days after inoculation. The conidia in the culture medium were collected with a dry method that consisted of sifting the culture medium through 0.5 mm mesh sieves.

Conidia viability was evaluated before the bioassays by re-suspending the conidia in Tween 80 solution (0.1% in H_2O, v/v) and placing a drop of this suspension onto complete medium for *Aspergillus nidulans* (Pontecorvo et al., 1953) with the following composition (per litre): 6 g $NaNO_3$, 0.52 g KCl, 1.52 g KH_2PO_4, 0.52 g $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, traces of $ZnSO_4$, 2 g peptone, 0.5 g yeast extract, 1.5 g hydrolyzed casein, 1 ml vitamin solution, 2.5 ml yeast nucleic acid hydrolysate, and 10 g glucose. To prepare solid media, 15 g agar was added and the pH was adjusted to 6.5. The preparation was incubated at 28 °C for 16 hours and the Petri dishes were then observed with a phase contrast microscope: a total of five hundred germinated and non-germinated conidia were counted; the percentage of viable conidia was determined.

2.2. Host preparation

Prepupal *A. fraterculus* larvae were obtained from the first generation of a colony reared in the laboratory by oviposition on papaya (*Carica papaya* - Caricaceae); the original flies were obtained from a field population collected from guava (*Psidium guajava*, Myrtaceae) fruit.

2.3. Disease cycle and histological studies

The fly larvae were placed on 25 cm wide × 5 cm deep × 30 cm long trays containing 3,000 cm^3 red latosol

mixed with 20% washed medium-texture construction sand sterilised in an autoclave for 40 minutes at one atmosphere. The soil was inoculated with 300 mg conidia containing 2×10^7 conidia/mg of known viability obtained as described above and incorporated into the soil, resulting in a distribution of 0.66×10^4 conidia/cm^3. This tray was used to expose the larvae to the conidia and infect them. Three hundred and fifty larvae were placed on the surface of the soil and left for 10 hours. By this time, all the larvae had penetrated deeply into the soil; groups of 100 larvae were collected by sieving the soil and transferred to the other three trays with 3,000 cm^3 autoclaved soil that was free of conidia to study the cycle of the disease histologically, for the PCR analyses and for mortality data. An additional tray with 3,000 cm^3 autoclaved soil free of conidia with 100 larvae that had not been exposed to the conidia was used as a control. All the trays were covered with a plastic gauze to exclude other insects. An evaluation of the percent reduction in the population was made in comparison to the control. All the experiments were conducted at 28 °C 75% soil moisture in a BOD with a photophase of 12 hours. The immatures were collected by sifting the soil at 24, 30, 48, 72 and 96 hours after infection by collecting 15 larvae and/or carcasses and 15 pupae from each tray, with three repetitions. Immediately after each collection, the material was prepared for histological evaluation and for entomopathogen DNA detection. The complete cycle of disease at the histological level was considered to be the time elapsed between contact of the larvae with the conidia on the inoculated soil and the appearance of larvae or pupae with externalised structures of the entomopathogen. All the procedures were repeated 3 times.

2.4. Histological evaluation

Whole larvae were fixed overnight in Carnoy's fluid. After fixation, the material was dehydrated in absolute alcohol for 30 minutes (three changes for 10 minutes each), in alcohol-xylene (1:1), alcohol-xylene (1:3), and xylene, and maintained in a paraffin bath at 60 °C for two hours. The specimens were individually embedded.

After embedding, the paraffin blocks were cut into 5-7 µm sections, deparaffinised and hydrated in decreasing alcohol concentrations, until attaining 100% water, and stained as follows: a) immersion in an aqueous solution of periodic acid for 5 minutes; b) washing with tap water for approximately 3 seconds; c) immersion in an aqueous solution of 0.1% basic fuchsin for 2 minutes, with gentle shaking; d) washing in running tap water for 30 seconds; e) immersion in acidified bisulfite; f) washing with tap water for 5 minutes; g) immersion in an aqueous solution (0.25% w/v) of light green for 1 minute with shaking; and h) washing with tap water. The histological sections were then dehydrated and mounted in Permount.

2.5. Detection of the entomopathogen DNA in the host using specific DNA primers

For detection and identification of the entomopathogen *Metarhizium anisopliae* var. *anisopliae* strain E9, in the immature stages (larvae and pupae) of the host, we used the primer ITSMet: (5' TCTGAATTTTTTATAAGTAT 3'), designed by Destéfano et al. (2004), with ITS4 (5' TCCTCCGCTTATTGATATGC 3') as a reverse primer. To carry out the DNA extractions, 15 larvae and pupae were first collected from the bioassay at 24, 30, 48, 72 and 96 hours; all treatments were repeated three times. Immediately after each collection, larvae and pupae were individually prepared for DNA extraction and for PCR analyses using the specific primer for entomopathogen identification. To avoid contamination by DNA from conidia that were not adhered to the external tegument of the host before DNA extraction, the hosts were carefully washed in 2.5% (v/v) sodium hypochloride solution for 3 minutes, and then in 0.1% (v/v) Tween 80 solution; they were then washed three times in 0.85% NaCl solution. The genomic DNA from conidia of the fungus and from the non-infected hosts (larvae and pupae) were initially extracted (Destéfano et al., 2004) and used in PCR analyses, in order to obtain amplification patterns for the DNA/PCR of the entomopathogen and the host. The PCR analyses using DNA extracted (Destéfano et al., 2004) from the larvae and pupae treated with conidia and collected from the bioassay at 24, 30, 48, 72 and 96 hours were run to search for the fungal DNA at the different stages of development of the disease: adhesion, penetration and colonisation of the host's body and development outside of the cadaver, as observed by histological methods. The PCR was conducted with ITSMet: 5' TCTGAATTTTTTATAAGTAT 3' as the forward primer and /ITS4 used as the reverse primer. The reaction (Destéfano et al., 2004) consisted of 3 minutes at 95 °C, followed by 32 cycles of 1 minute at 94 °C, 1 minute at 46 °C and 1 minute at 72 °C, with a final extension of 3 minutes at 72 °C. The products were visualised under UV light in 1% agarose gels stained with ethidium bromide.

3. Results and Discussion

Metarhizium anisopliae conidia were found to cause disease and death of *A. fraterculus* larvae in a bioassay simulating field application. This simple bioassay could be used to determine the mean lethal concentration of conidia (LC_{50}) as well as the mean time to death (LT_{50}) in the evaluation of *M. anisopliae* as an agent for microbial control of fruit flies. The life cycle of the host insect from the pre-pupal stage until adult emergence on soil without pathogens lasted approximately 12 to 17 days (considered as the beginning of adult emergence) at an ambient temperature of about 28 °C and a soil humidity of about 75%, similar to what was found by Orlando and Sampaio (1973), who reported a cycle of 10 to 20 days. The complete cycle under laboratory conditions is 30 days from egg to adult (Morgante, 1991). The complete cycle of disease induced by conidia with 90% viability lasted 96 hours under the bioassay conditions. The disease cycle begins with conidia contacting and adhering to the integument of the host (Figure 1), followed by germination, appressorium production (Figure 2a) or without appressorium formation

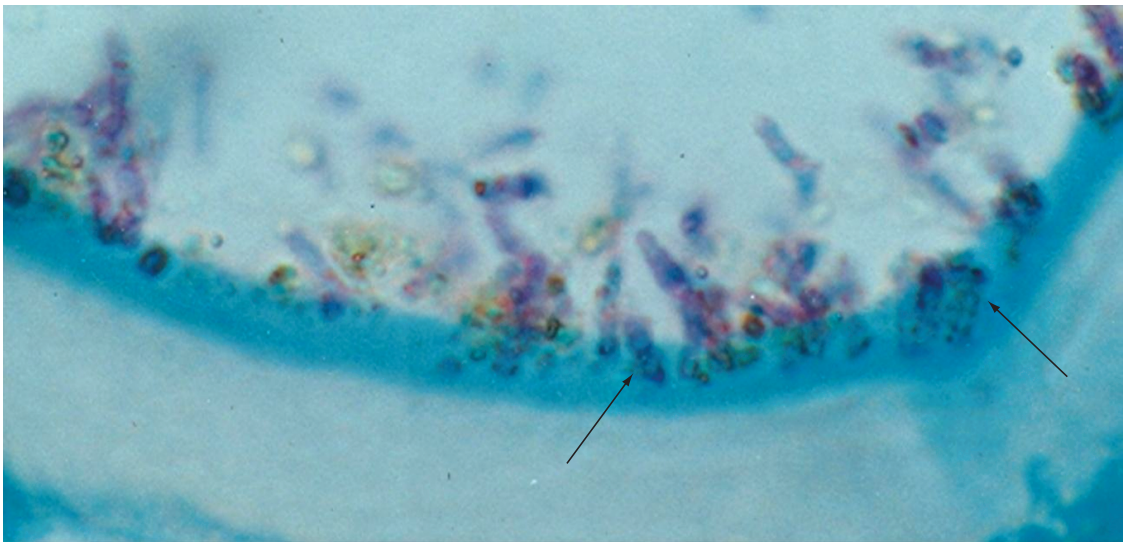

Figure 1. First phase of the development of disease on the epicuticle of the host, due to contact with the conidia, which have not yet germinated. They are reddish in colour due to the histological preparation; the epicuticle is stained bluish.

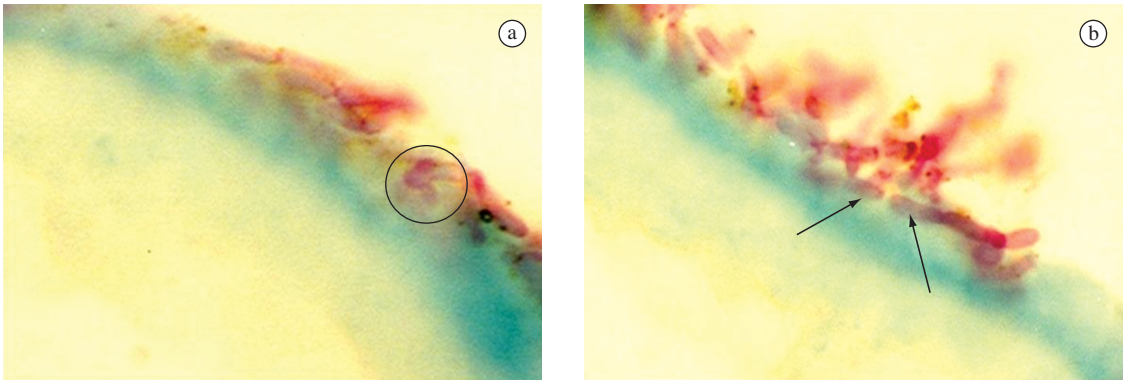

Figure 2. The already-germinated conidia have initiated adhesion, and penetration occurs in two different ways: a) formation of the appressorium (marked with a circle); or b) germination and growth towards the interior part of the tegument, without formation of the appressorium (arrows).

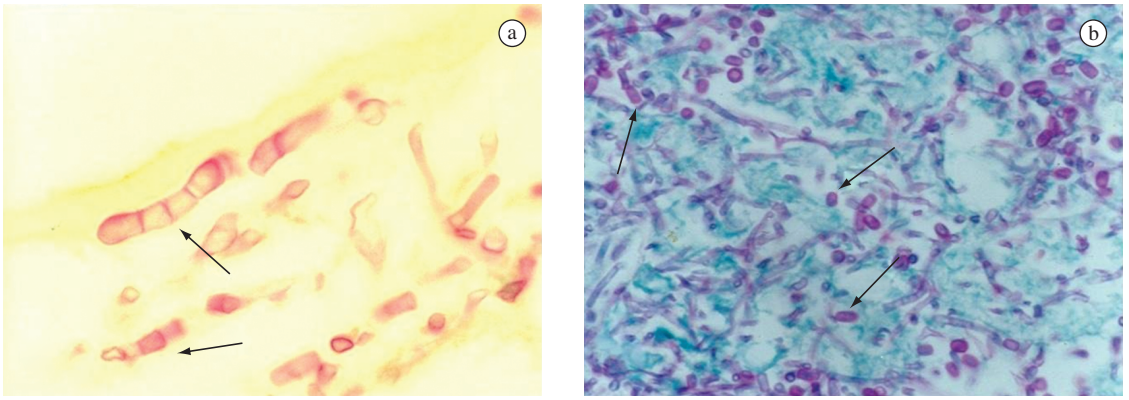

Figure 3. A dimorphic phase begins within the host: a) septation of the hifae; and b) development of a yeast-like form, with widespread proliferation within the interior of the host (marked by arrows).

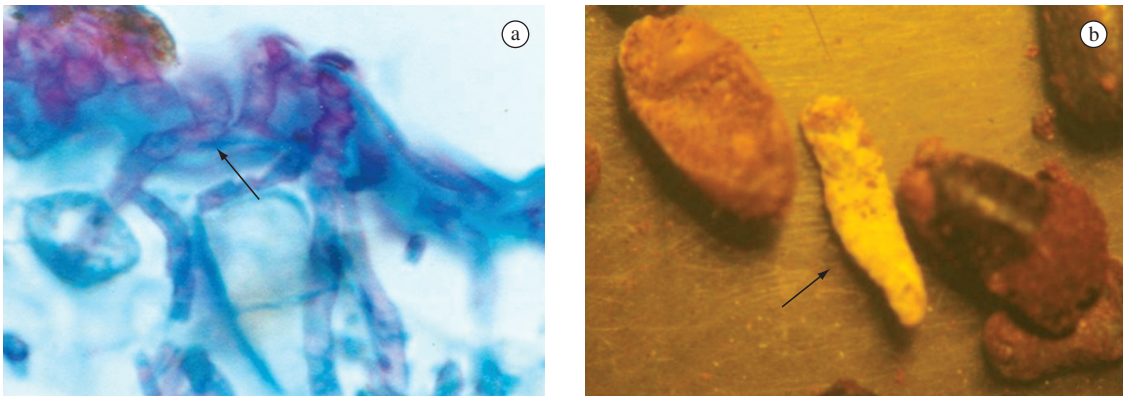

Figure 4. After development and taking over the interior of the insect, exteriorisation of the entomopathogenic structures begins: a) dimorphism of the yeast cell to the mycelial form; b) culminating in the development of mycelial structures (like cotton) on the external tegument of the larva, as indicated by the arrow.

(Figure 2b), penetration (Figure 3a,b), development in the hemocoel with the dimorphic (yeast-like forms) stage represented by the formation of short, thick and septated hyphal bodies, mostly with one or more septation (Figure 3a), to mycelial exteriorisation (Figure 4a,b) and as shown (Figure 6 a,b) on pupae mycelial exteriorisation and conidiogenesis on the carcass. The infected host insect can survive until adult stage and in this case the complete cycle of the disease occurs with a very dense production of conidia (Figure 7) the infective units, produced on the cuticle covering the cadaver. The time sequence of events from contact to host death was estimated because the phases cannot be determined with precision due to the dynamism of the process; they may be described as follows: a) infection by adherence of conidia to the cuticle and penetration, when a germ tube penetrates the cuticle of the host, or formation of the appressorium is observed, which occurs during the first 24 hours after the larvae are placed on the surface of the soil treated with conidia; after this time, most larvae are already in the pupal stage. When adhesion occurs, formation of the appressorium (Figure 2a circle) seems to be of fundamental importance for the infective mechanism in some fungi. However, penetration may also occur by elongation of the hyphal tip (Figure 2b) through the cuticle of the host; as we also observed in this kind of mycelial growth without hyphal differentiation into the appressorium. Therefore, there are two different routes of penetration. This has also been described by Zacharuk (1970a, b) for *M. anisopliae* infecting Elateridae (Coleoptera) larvae, by Pekrul and Grula (1979) for *Beauveria bassiana* infecting *Heliothis zea*. Therefore, formation of the appressorium seems to be the rule during the penetration phase of *M. anisopliae* and other fungi. St. Leger et al. (1989) observed the in vitro production of an appressorium in *M. anisopliae*. Magalhães et al. (1990) also observed this in the fungus *Zoophthora radicans* (Zygomycetes, Entomophthorales), demonstrating that an appressorium can be produced both in vivo and in vitro. It could be a facultative structure in the infection process, with induction being affected by factors such as chemical constitution or nutrients present in the exudate or on the cuticle of the host; more studies will be needed to clarify this point. Penetration begins following adhesion to the cuticle; after 30 hours it is completed.

After 30 hours, already inside the host, the propagule of the entomopathogen appears in a yeast-like form; these are blastospore-like structures, demonstrating developmental dimorphism after the filamentous phase on the outside of the cuticle. The dimorphic phase is present during the entire fungal infection process (Orlowski, 1991). It occurs in animals, including humans, during the fungal pathogenic process (Szaniszlo et al., 1985; Wang and Szaniszlo, 2007). This dimorphism is probably important to overcome host-defense mechanisms during the colonisation phase. The dimorphic phase can be induced by CO_2, pH, osmotic pressure and chemical constitution, as well as other factors such as vitamins, nitrogen, amino acids, and in many cases it is induced by cystein (Bartnicki-Garcia and Nickerson, 1962; Brown, 1965; Bartnicki-Garcia and Lippman, 1972). Insect haemolymph consists of a fluid plasma with various types of blood cells. The plasma contains various inorganic ions, in many different concentrations; there are large differences in organic constituents among species. Osmotic pressure of the hemolymph in many insects is about seven to eight atmospheres; but it can reach 12 atmospheres in some species and stages. In *A. fratercullus* it is about 9.27 atmospheres, with a pH of 6.7, in the prepupal stage (Schneider, 2002). Production of blastospores varies with strain; it is also influenced by pH, which ranges from about 6.5 to 8.0. The best blastospore yields in in vitro cultures of three different strains of *M. anisopliae* were obtained at 30 ºC by Kleespies and Zimmermann (1992). The duration of the dimorphic phase varies with strain; induction and duration are influenced by haemolymph parameters, depending on the host species and stage of development (Schneider, 2002). The morphology of these structures blastospores allows them to colonise the body of the host, since circulation in the hemocele

is facilitated, with consequent invasion of other tissues. Given the relevance of this phase for disease development, it deserves more detailed studies of the interaction with host defense mechanisms.

The mycelial phase begins after 48 hours; during the mycelial phase, hyphal bodies develop from inside to outside the host's carcass, with the appearance of white cotton-like mycelia after 96 hours, culminating with the conidia completely covering the cadaver when the disease cycle is completed (green muscardine phase), as is illustrated in Figure 6b. The duration of each phase depends on the microorganism and the host; conidia germination Oliveira and Messias (1996) and conidia formation on the cadaver can be directly influenced by abiotic factors, such as temperature and ultraviolet light (UV), mainly UV-B. Silveira and Messias (1982), studying the same fungal strain, reported a cycle of disease development of 90 hours, when the hosts were 3rd-instar nymphs of *Deois flavopicta* (Homoptera: Cercopidae), the pasture spittlebug. Biotic characteristics of the pathogen and the host interact during each developmental phase. The pathogen requires conidia germination on the host's cuticle, where it will encounter the first reaction to penetration. Messias and Pupin (1990) and Messias (1992) detected in vitro fungistatic activity against germination and development of *M. anisopliae* caused by caprylic and caproic acids in cuticle extracts of *Diatreae saccharalis* (Leptdoptera: Pyralidae) larvae, the sugar cane borer. The occurrence of blackened melanised pupae as shown in Figure 5, this kind of reaction to infection can occurs on larvae and pupae without development of the entomopathogen, or of opportunistic microorganisms, is another factor antagonising penetration, but this phenomena seems to be a very strong reaction of the host to avoid infection by the pathogen. Based on in vitro studies made with *Ceratitis capitata*, the Mediterranean fruit fly, Marmaras and Charalambidis (1992) and Marmaras et al. (1993) showed that melanisation as a mechanism of defense against the attack of *Escherichia coli* is due to the action of tyrosinase, which generates reactive intermediate quinone compounds that bind to the tegument protein. St. Leger et al. (1991) and St. Leger (1993) cited melanisation as a selective factor in terms of the virulence of entomopathogenic fungi. We observed the same evidence in our study, at the initial stage of colonisation.

Figure 5. Melanised pupae.

Thus, under bioassay conditions, simulating normal application levels, we demonstrated that *M. anisopliae* conidia can cause infection, disease and death in *A. fratercullus* larvae at the prepupal stage. Also, the use of histological techniques based on simple light microscopy methods allowed in situ observation of the infective process and of the production of the various entomopathogen structures during each phase of disease development. This may permit histochemical evaluation of this relationship, including the dimorphic phase, contributing to the identification of the physicochemical interaction between pathogen and host during disease development, including morphological and epigenetic aspects that occur during the dimorphic phase.

3.1. Detection within the host using a specific DNA primer

PCR evaluation with a specific primer conducted at each stage of the disease permitted us to trace the fungus DNA on the cuticle and within the host, confirming the presence of the entomopathogen strain E9 responsible for infection and death of the host. PCR of the entomopathogen DNA in the host insect was run for each group of 15 larvae or pupae. Observation in the larvae was only possible for

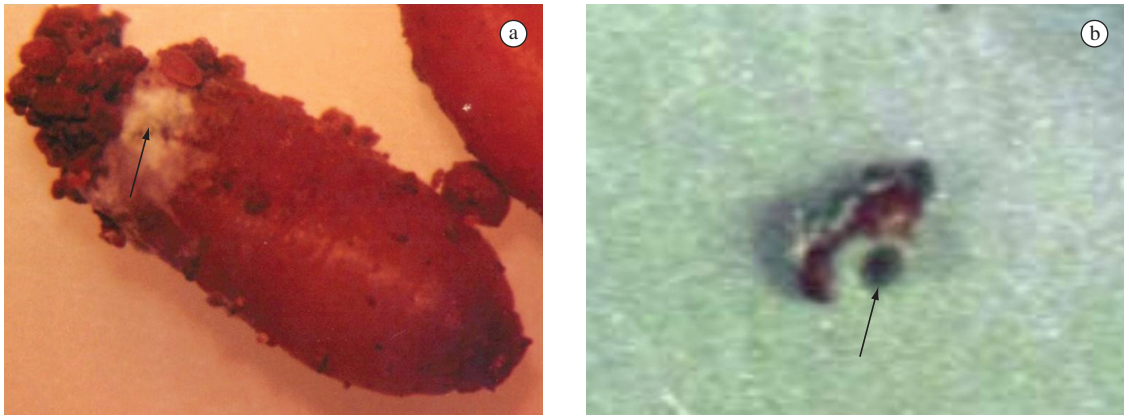

Figure 6. Development of the structures and the propagules of the entomopathogen: a) pupa, the arrow indicates mycelia (like cotton) growth; and b) pupa showing conidia in the green area indicated by the arrow head.

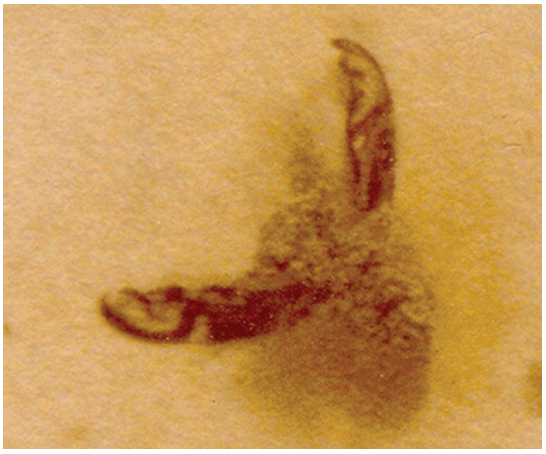

Figure 7. Development of the structures and the propagules of the entomopathogen on the surface of the dead adult inset.

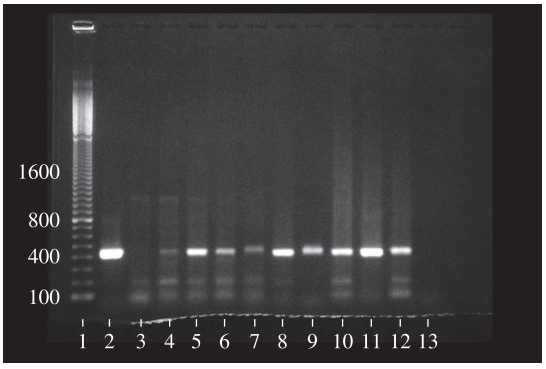

Figure 8. Detection of entomopathogen DNA during the different developmental stages of the disease, using specific primers: 1) 100bp ladder; 2) E9 genomic DNA; 3) larvae of the host *A. fraterculos* not exposed to conidia at time T0; 4) larvae exposed to conidia at T0; 5) larvae 24 hours after inoculation; 6) pupa 24 hours after exposure to conidia; 7) larva 48 hours after exposure to conidia; 8) pupa 48 hours after exposure to conidia; 9) larva 72 hours after exposure to conidia; 10) pupa 72 hours after exposure to conidia; 11) larva 96 hours after exposure to conidia; 12) pupa 96 hours after exposure to conidia; and 13) control preparation used as a vehicle for the treatments.

those that died within the first 30 hours, since after this interval, the larvae had either died or were already in the pupal stage; the larval infection rate was 30%. Within the group of 15 pupae, the DNA was extracted individually, demonstrating infection at all time points in about 33% of the pupae; in the histological exam, this detection was 30%, indicating that even if the larvae transformed into pupae, they had already been infected and would die in the pupal stage. Figure 8 shows the results obtained by PCR in the different phases of the disease cycle.

The reduction in population in the control soil tray (no conidia) was 37%; this value was obtained by subtracting the number of emerging adults from the number of larvae exposed in the tray. Histological and molecular exams gave similar percentages of mortality, indicating that both methodologies can be used for evaluating mortality, although the molecular methodology was found to be easier and quicker. However, further studies should be carried out to determine mortality parameters. The PCR methodology proved to be a rapid and efficient way to study *M. anisopliae* infection of *A. fraterculus* during immature stages, since it was sensitive enough to detect fungus DNA at all stages of development of the disease in single larvae and pupae. In the histological examination, a dense development of propagules of the entomopathogen occurred in the hemocoel 48 hours after infection. Though a band was already detected by PCR within the first 24 hours, indicating presence of the entomopathogen DNA, it was confirmed with more certainty after 48 hours, since at this time the larvae could already be dead at the larval or pupae stage, indicating that infection probably occurred during the larval stage and that the fungal propagules developed during pupal development. It is important to note that neither contact with the fungal conidia nor infection of the larvae by the fungus at this initial stage impeded development into pupae. This indicates that evaluation with a specific primer is more precise when carried out at 48 hours after exposition to the entomopathogen conidia. This would be the minimum time for determining how much of the population is affected by application of the entomopathogen, although this conclusion should be confirmed in the field.

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