

MECHANISM OF INFECTION AND COLONIZATION OF *RHIPICEPHALUS SANGUINEUS* EGGS BY *MERTARHIZIUM ANISOPLIAE* AS REVEALED BY SCANNING ELECTRON MICROSCOPY AND HISTOPATHOLOGY

Marcos Valerio Garcia¹; Antonio Carlos Monteiro^{1*}; Matias Juan Pablo Szabo²; Nancy Prette¹; Gervásio Henrique Bechara²

¹Laboratório de Microbiologia, Departamento de Produção Vegetal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP, Brasil; ²Departamento de Patologia Animal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, SP, Brasil.

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ABSTRACT

The present work aimed to verify the penetration pattern of the *Metarhizium anisopliae* fungus into *Rhipicephalus sanguineus* tick eggs, as well as the lesions occurred inside the egg. The fungus adherence and penetration were studied by scanning electron microscopy and the action of fungus in the internal tissues was evaluated through conventional histological sessions. For the observation of these events, experimental infections were performed in 11 groups of *R. sanguineus* eggs containing 25 mg each. The eggs were bathed during 3 minutes under manual shaking in a 10⁸ conidia/mL suspension. The bath was performed only in the suspension vehicle for the control groups. The eggs were processed for histopathological analysis and scanning electron microscopy at the following times after infection: 1 and 18h and one, two, three, four, five, six, seven, nine and eleven days. Relevant conidial germination was observed in 67% of eggs 18h after inoculation and fungus penetrated in 92.6% of eggs 5 days after the infection. The pathogen extrusion occurred in 87% of eggs 7 days after infection, reaching 100% at the 9th day. In the histopathological analysis, no lesions worthy of record was observed, however, it should be emphasized that significant reduction (53.9%) of hatching from infected eggs was observed.

Key words: biological control, microbial control, entomopathogenic fungus, ticks

INTRODUCTION

Rhipicephalus sanguineus, a cosmopolitan tick is probably the tick of greatest worldwide distribution (17), and represent the sole species of the genus in Brazil (2). Its description has been frequently associated to the presence of its host dog (1,19,10,21), being adapted to pets in urban centers. *R. sanguineus* is a vector of several relevant pathogens for dogs, including agents of babesiosis, haemobartonellosis, hepatozoonosis and ehrlichiosis, some of them potentially transmissible to man. (8,22). The control of this species of tick

as a way of establishing better sanitary conditions to pets in general, remarkably to dogs is vital.

The control is currently performed by means of synthetic chemical products (acaricides). Recently, the resistance development of *R. sanguineus* to some active principles used in these formulations was verified (12). One of the alternatives to the use of acaricides is the biological control, especially with fungi, that showed to be an effective and promising technique, as previously verified (6,13,15,16). *R. sanguineus* larvae bathed in suspensions with progressive concentrations of conidia of *Beauveria bassiana* demonstrated a decrease on the molt

*Corresponding Author. Mailing address: Laboratório de Microbiologia, Departamento de Produção Vegetal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Via de Acesso Prof. Paulo Donato Castellane, s/n. 14884-900, Jaboticabal, SP, Brasil. Tel.: (+5516) 3209-2652; Fax: (+5516) 3203-4275; E-mail: montecar@fcav.unesp.br

percentage (3). The concentration of 10^8 con./mL of *B. bassiana* and *M. anisopliae* caused the death of 95% of larvae of *R. sanguineus* 20 days after infection (14), and *M. anisopliae* applied at different tick stadiums caused high mortality rate in all unfed instars (20). *B. bassiana* caused a reduction in percent hatching of larvae derived from infected eggs and in percent ecdysis of larvae and nymphs of *R. sanguineus* (18).

Only a few studies were conducted in order to assess the infection mechanisms of different phases of ticks life cycle by fungi, including eggs. The penetration and colonization of *R. sanguineus* engorged female by *M. anisopliae* was assessed (9), but there is a lack of information and knowledge with regard to the phenomenon or events concerning the pathogenicity mechanism of fungi on ticks. This work aimed to demonstrate the adhesion, germination, penetration and colonization of eggs of *R. sanguineus* by *M. anisopliae* as well as the lesions caused by fungus in the tick's egg.

MATERIALS AND METHODS

The E9 isolate of *Metarhizium anisopliae* obtained from *Deois flavopicta* (pasture spittlebug) was used, kept in stock culture in the Microbiology Laboratory of the Vegetal Production Department Faculty of Agrarian and Veterinary Sciences (FCAV) - São Paulo State University (Unesp), Jaboticabal City, São Paulo State, Brazil. The isolate was cultivated in Petri dishes containing potato-dextrose-agar medium (PDA), incubated at 27°C in the absence of light, for approximately 12 days.

The tick eggs collected until the 7th day after the beginning of posture were obtained from the *R. sanguineus* colony kept in the Immunopathology Laboratory of the Animal Pathology Department, FCAV– Unesp. For the colony maintenance, the ticks were fed on rabbits free of previous contact with ticks. During feeding, the ticks were restricted to the hosts by transparent plastic chambers (4) fixed with non-toxic material (Britannia Adhesives P4104 Latex, England) to its depilated back. For the molt of the engorged instars as well as for its simple maintenance, the ticks were kept in an incubator at temperature of 28°C and relative humidity above 80%.

For the infection, a suspension prepared in a 0.1% (v/v) Tween 80® solution was used at the concentration of 10^8 con./mL (13,14,15,16,18), obtained from young colonies of fungus (conidial viability: 97.6%). The *R. sanguineus* eggs were divided into 11 groups composed of 25 mg each, in glass tubes. Each group was bathed with 1 ml of the conidial suspension during 3 minutes, the excess liquid was removed using filter paper and the glass tubes were kept in incubator at 27°C, absence of light and relative humidity above 80%. The infected eggs were collected in 1 and 18h and one, two, three, four, five, six, seven, nine and eleven days after treatment. In each time, a control group, bathed only with the suspension vehicle and another control group, no treated were also collected. After collecting

in each pre-established time, the treated eggs were divided into two parcels: one addressed to the histopathological preparing and the other for the scanning electron microscopy.

The material addressed to the histopathological analysis was immersed in buffered formaldehyde solution for 12 to 24h. The fixed material was submitted to paraffin inclusion according to the histology routine techniques. Later, serial longitudinal cuts were performed in order to obtain the sections representative of both the infected egg and the control tissues. The sections were stained with hematoxylin-eosin stain for visualization of the tissue general alterations and Gomori Metanamine Stain (GMS), specific for fungi. For the histopathological analysis, only egg samples fully preserved were considered (morphologically intact).

The eggs addressed to the scanning electronic microscopy analysis were fixed in glutaraldehyde at 3%, in potassium-phosphate buffer at 0.05M and pH 7.4 for 72h. The eggs were washed in pure buffer solution for six consecutive times each 20 minutes. Later, the eggs were dehydrated in ethylic alcohol, dried in a CO₂ critical point drier, recovered with gold particles (35 nm) and electronmicrografies were made in JEOL JSM 5410 scanning electron microscopy, operating at 15 kV (10).

RESULTS AND DISCUSSION

The histopathological analysis showed no conidia adhered to the eggs membrane in the period of 1h after infection (Table 1) (Fig. 1 A). One may suppose that the conidia were washed away from eggs during samples processing. The smooth surface

Table 1. Absolute and percent values of eggs of *Rhipicephalus sanguineus* presenting adhesion, germination, penetration and extrusion of *Metarhizium anisopliae*, according to the post-infection time. Values between parenthesis indicate the total number of samples (eggs) analyzed.

| Post-infection Time | Adhesion (%) | Germination (%) | Penetration (%) | Extrusion (%) |
|---------------------|--------------|-----------------|-----------------|---------------|
| 1 hour (40) | 0 | 0 | 0 | 0 |
| 18 hours (12) | - | 8 / 67% | 0 | 0 |
| 4 days (20) | - | - | 16 / 80% | 0 |
| 5 days (27) | - | - | 25 / 92.6% | 0 |
| 7 days (23) | - | - | 23 / 100% | 20 / 87% |
| 9 days (16) | - | - | - | 16 / 100% |

of eggs and successive washings during histopathological processing may have contributed to such condition.

In test and control groups submitted to the histopathological analysis, no lesions worthy of record or embryonic phase the eggs were observed. However, in the control group where eggs were not processed for histopathology analysis and scanning electron microscopy, a high hatching (95%) was observed, while in eggs from the test group, a relevant reduction of hatching (53.9%) was observed. These observations suggest that morphological alterations (lesions) in eggs from the test group occurred after 11 days. Biochemical and physiological alterations may have occurred during the period analyzed, however, with no morphological manifestation perceptible by microscopy.

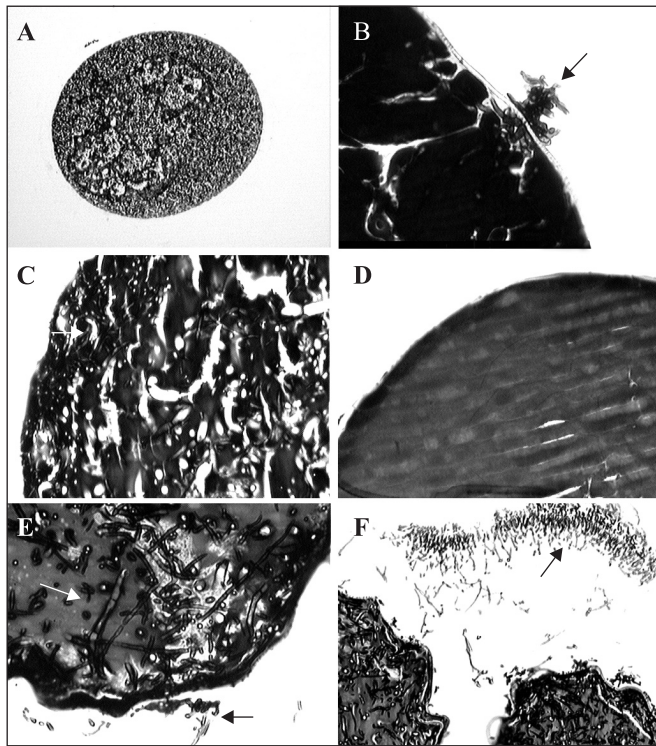


Figure 1. Photomicrographs of eggs of *Rhipicephalus sanguineus* tick in several times after experimental infection with conidial suspension of *Metarhizium anisopliae*. A – absence of conidia on the egg surface 1h after infection (G.M.S, magnificence of 10x); B – fungus penetration into egg (pointed by arrow) 4 days after infection (G.M.S, magnificence of 40x); C – egg colonization (pointed by arrow) 5 days after infection (G.M.S, magnificence of 40x); D – intact internal part of egg from control group (G.M.S, magnificence of 40x); E – egg colonization (pointed by arrow) and fungus extrusion (pointed by arrow) (G.M.S, magnificence of 40x) 9 days after infection; F – external development and conidiogenesis of fungus (pointed by arrow) in egg 9 days after infection (G.M.S, magnificence of 40x).

Report regarding susceptibility of eggs and larvae from *B. microplus* tick to the fungus *M. anisopliae* ratify a reduction on the hatching percentage that ranged from 3.3 to 66%, according to the isolate and the fungus concentration used (5).

Observations performed 18h after infection showed that 67% of infected eggs presented conidia germinated on their surface. Four days after infection, 80% of infected eggs were already colonized by fungus, reaching 92.6% at the 5th day. The pathogen extrusion was observed 7 days after infection, when 87% of eggs presented the phenomenon, reaching 100% at the 9th day, with a large amount of conidia adhered to the external surface of host (Table 1).

The fungus penetration inside the egg and the colonization were observed at the 4th and 5th days after infection. Fig. 1 B emphasizes the pathogen penetration into the egg membrane

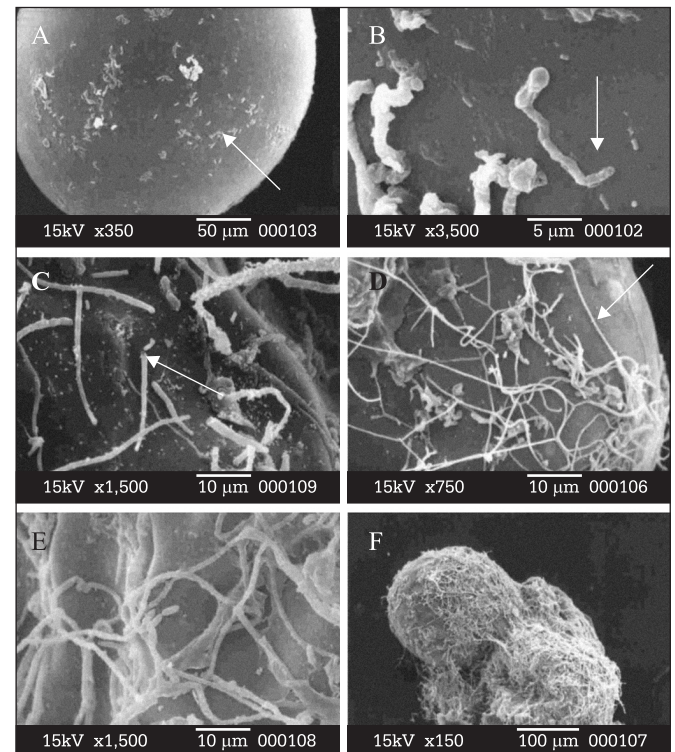


Figure 2. Scanning electronmicrografies of eggs from *Rhipicephalus sanguineus* tick in several times after experimental infection with conidial suspension of *Metarhizium anisopliae*. A – conidia adhered to egg membrane (pointed by arrow) 1h after infection; B – germination and apparent aplanospore formation (pointed by arrow) 18h after infection; C and D – pathogen extrusion (pointed by arrow) and formation of hyphal bodies on the external egg surface (pointed by arrow) 4 and 5 days after infection; E and F – external development and sporulation of fungus on the egg surface.

and Fig. 1 C shows the egg fully colonized with a large amount of hyphal bodies. In samples from control group collected at the same time after infection, the presence of fungus inside the egg was not observed (Fig. 1 D). The histological cuts performed in eggs collected 9 days after infection emphasized the pathogen extrusion and conidiogenesis (Figs. 1 E and F).

The electronmicrografies of the infected eggs showed a large amount of conidia adhered to the external surface of eggs in a diffuse way (Fig. 2 A) 1h after infection. The conidium germination and the formation of the germinative tube were evidenced through electronmicrografies performed 18h after infection (Fig. 2 B). Only few works, showing by means of scanning electron microscopy, the conidium adhesion and germination and the apressoria formation, can be found in literature, corroborating that *M. anisopliae* penetrates into *B. microplus* (7) and *R. sanguineus* (9) through the cuticle. The electronmicrografies performed at the 4th and 5th days after infection showed the pathogen extrusion and wide hyphal bodies on the egg surface (Figs. 2 C and D). The conidiogenesis occurred around the 9th day after infection, being observed along the entire extension of eggs (Figs. 2 E and F).

M. anisopliae is, in *in vitro* tests, pathogen of eggs of *R. sanguineus*. The conidium germination as well the penetration into the cuticle occurred quickly. The fungus colonizes massively the host internal tissues causing its death within a few days and consequently the non hatching of larvae. Intense micelial growth and high production of conidia on the egg surface complete the fungus development cycle.

RESUMO

Mecanismo de infecção e colonização de ovos de *Rhipicephalus sanguineus* por *Metarhizium anisopliae*, revelado por microscopia eletrônica de varredura e histopatologia

O presente trabalho teve como objetivo verificar a forma de penetração do fungo *Metarhizium anisopliae* em ovos do carrapato *Rhipicephalus sanguineus*, assim como as lesões infringidas no interior do ovo. A aderência e penetração do fungo foram estudadas por meio da microscopia eletrônica de varredura e a ação do fungo nos tecidos internos avaliada em seções histológicas convencionais. Para observação destes eventos, realizaram-se infecções experimentais em 11 grupos de ovos do *R. sanguineus* contendo 25 mg cada. Os ovos foram banhados durante 3 minutos, sob agitação manual, em suspensão com concentração de 10⁸ conídios/mL. Nos grupos controle o banho foi realizado apenas no veículo da suspensão. Os ovos foram processados para análise histopatológica e microscopia eletrônica de varredura nos seguintes tempos após a infecção: 1 e 18h, e um, dois, três, quatro, cinco, seis, sete, nove e onze dias. Observou-se grande germinação de conídios

em 67% dos ovos 18h após a inoculação e o fungo penetrou em 92,6% dos ovos 5 dias após a infecção. A extrusão do patógeno ocorreu em 87% dos ovos 7 dias após a infecção, chegando a 100% no 9^o dia. Nas análises histopatológicas não foram observadas lesões dignas de nota, porém deve-se ressaltar que houve significativa redução (53,9%) na eclosão a partir dos ovos infectados.

Palavras-chave: controle biológico, controle microbiano, fungo entomopatogênico, carrapato

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