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CROP PROTECTION

Infection of silkworm larvae by the entomopathogenic fungus *Metarhizium anisopliae*

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ABSTRACT: *The isolate E9 of **Metarhizium anisopliae** was used in commercial hybrids of **Bombyx mori** larvae to evaluate its biological effect. Symptomatological analyses showed typical signs of fungal infection. Histopathology revealed the presence of large numbers of hemocytes in the hemocoel, and on the sixth dpi the bodies of the insects appeared to be colonised by the fungus. The isolate E9 is pathogenic to larvae **B. mori** and; therefore, death of the insects was caused by the colonization of fungus in the epidermal and mesodermal tissues.*
Key words: ***Bombyx mori**, histopathology, **Metarhizium anisopliae**, fungal infection.*

Infecção de lagartas do bicho-da-seda pelo fungo entomopatogênico *Metarhizium anisopliae*

RESUMO: *O isolado E9 de **Metarhizium anisopliae** foi usado em larvas* híbridas *de **Bombyx mori** para avaliar seu efeito biológico. A sintomatologia revelou sinais típicos de infecção por fungos. Na histopatologia foi verificado um aumento no* número de *hemócitos, sendo que, no 6 dpi, todo o corpo do inseto se apresentou colonizado pelo fungo. O isolado E9* é patogênico para *lagartas de **B. mori**, causando sua morte pela colonização dos tecidos de origem epidérmica e mesodérmica.*
Palavras-chave: ***Bombyx mori**, histopatologia, **Metarhizium anisopliae**, infecção fúngica.*

Metarhizium anisopliae is an entomopathogenic fungus that is capable of adapting to heterogeneous media and it infects a wide variety of insects primarily due to the evolution of genes which cause the degradation of the host cuticle, detoxification and biosynthesis of toxins (GAO et al., 2011). ***M. anisopliae*** naturally infects over 300 species of insects (ALVES, 1998), as well as the silkworm ***Bombyx mori*** (Lepidoptera: Bombycidae), which is an insect model in scientific studies and has economic importance due to its role in silk production (STAYKOVA et al., 2012).

Entomopathogenic fungi act via contact and they require some environmental conditions to germinate on insects. Such conditions are reported in the ***B. mori*** creation room, which increases the infection and favours the multiplication and spread of the pathogen. Some previous studies conducted in sericulture regions of Brazil and other countries have shown fungal incidence in ***B. mori*** larvae (ALVES, 1998; KUMARI et al., 2011). However, data that correlate symptoms and histopathology of infection are scarce.

Considering the economic importance of ***B. mori***, a better understanding and description of fungal infection process may help to manage the risks of disease spreading in silk production. Silkworms are widely used as a model for cellular immunity response studies in insect-pathogen interactions, even though there is a lack of information about histopathological studies. Consequently, this studied analysed the symptomatology and histopathology of ***B. mori*** larvae inoculated experimentally by ***M. anisopliae***.

The fungus ***M. anisopliae***, E9 strain, was obtained from ***Deois flavopicta*** (Hemiptera: ***Cercopidae***) and supplied by EMCAPA - Capixaba Research Company Agropecuaria. It was cultured in Petri dishes with a complete medium for conidial production (agar 20g, yeast extract 5g, KH_2PO_4 0.36g, Na_2HPO_4. $7H_20$ 1.05g, $MgSO_4.7H_2O$ 0.60g, KCl 1.00g, $NaNO_3$ 1.58g, glucose 10g and distilled water 1000mL), which was previously autoclaved and incubated at 26°C with a 12 hour photoperiod for 10 days (ALVES, 1998). The conidia were then collected by scraping the surface of the medium

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and suspended in a solution of distilled water and 0.01% Tween 80®. After quantification, using a Neubauer chamber, a suspension was prepared at a concentration of 1.5×10^8 conidia mL^{-1}.

Third instar ***B. mori*** larvae were obtained from the BRATAC S/A silk company; they were kept in polyethylene boxes at room temperature and humidity and fed with fresh mulberry leaves twice daily until the fifth instar.

At the beginning of the fifth instar, 40 larvae were quickly immersed for 5 seconds in the conidia suspension. An identical control group was immersed in a solution of distilled water plus 0.01% Tween 80®. Larvae were subsequently placed in boxes and fed with mulberry leaves, as described above. The signs and symptoms of the disease were assessed daily, based on ALVES (1998), from the first to the eleventh day post-inoculation (dpi) and the dead larvae were transferred to a Petri dish with its bottom covered with filter paper (moist chamber) to promote the extrusion of the fungus, which confirmed the mortality of the larvae. The experiment was conducted in triplicate.

Two larvae from each group (control and inoculated) were anesthetized with ether and fixed in Bouin for 24 hours. This procedure was performed daily from the first to the seventh dpi. After fixation, the larvae were rinsed in 70% alcohol, processed for routine histological, and stained with hematoxylin and eosin for analysis of general morphology, and Gomori methenamine technique (GROCOTT, 1955) for fungi.

Evidence of infection of ***B. mori*** larvae by ***M. anisopliae*** was observed externally from the second dpi. The infected larvae decreased the size of the food that was available, had smaller bodies than insects in the control group, and began to present darkened spots in the integument. On the third dpi decreased activity was evident with dry stools and integument with a wrinkled appearance. Between the fourth and fifth dpi browning was evident with the appearance of dark spots on the surface, especially in the regions of the intersegmental membranes and spiracles. On the sixth dpi the larvae began to die and on the seventh dpi all the larvae were dead. These dark spots corroborated previous studies (ALVES, 1998; TOLEDO et al., 2010) and confirmed what is known about the integument of insects being the main route of entomopathogenic fungal entry, including ***M. anisopliae***; the dark spots were the result of fungal germination and penetration in the cuticle through the action of extracellular enzymes and mechanical pressure exerted by ***hyphae***.

In the hemocoel, hyphal bodies grow profusely and multiply by budding of pre-existing cells, the blastospores, as observed by KUMAR et al. (2004) in ***B. mori*** infected with ***A. flavus***, and by GAO et al. (2011) in a comparative analysis of the genome and the broad-spectrum insect pathogen of ***M. anisopliae*** and ***M. acridum***.

On the sixth dpi, ***hyphae*** forming a mycelia complex in the fatty tissue, integument, digestive tract, silk gland, reproductive organs, muscle and trachea (Figure 1A-F). Furthermore, during this period microscopic image showed little injury to the internal tissues, as reported by KUMAR et al. (2004).

A large development of mycelium was observed in the mid gut. This was related to the availability of nutrients in tissues because, as pointed out by TOLEDO et al.(2010), nutrient limitation leads to the formation of ***hyphae*** with a smaller diameter. Fatty tissue is another preferential target. Figure 1F shows that we detected the presence of ***M. anisopliae hyphae*** and blastospores in the lumen of the tracheal system. Tracheae are responsible for aeration and; therefore, form channels of oxygen transport which, in this case, can act as an important distribution system of the pathogen since the spiracles are also sites of pathogen penetration (TOLEDO et al., 2010). Furthermore, SCHRANK & VAINSTEIN (2010) state that the blastospores facilitate dispersal of the fungus, thereby causing generalized infection.

After the seventh dpi all the ***B. mori*** larvae were dead, mainly because of the action of mycotoxins and mechanical blockage of the digestive tract. Death of insects was following by major branches of the fungus through the ***B. mori*** epidermal and mesodermal tissues, with saprophytes growing on the corpse. Mycelium takes the place of the internal organs and; therefore, soon after death ***B. mori*** has a normal appearance (GAO et al., 2011).

The mummification of bodies of ***B. mori*** occurred between the eighth and ninth dpi; during this period ***M. anisopliae*** emerged through the integument of the ***larvae***, by mechanical pressure, especially in the intersegmental regions, which offered less resistance to the fungus (KUMAR et al., 2004). Subsequently, aerial ***hyphae*** were formed that originated branched conidiophores which were disseminated in the environment (ALVES, 1998).

In the colonization of the ***B. mori*** integument, hyphae of ***M. anisopliae*** passed through the basal lamina and reached the basal surface of epithelial cells between the third and fourth dpi. Tissue destruction was easily observed from the fifth dpi (Figure 2A) due to the

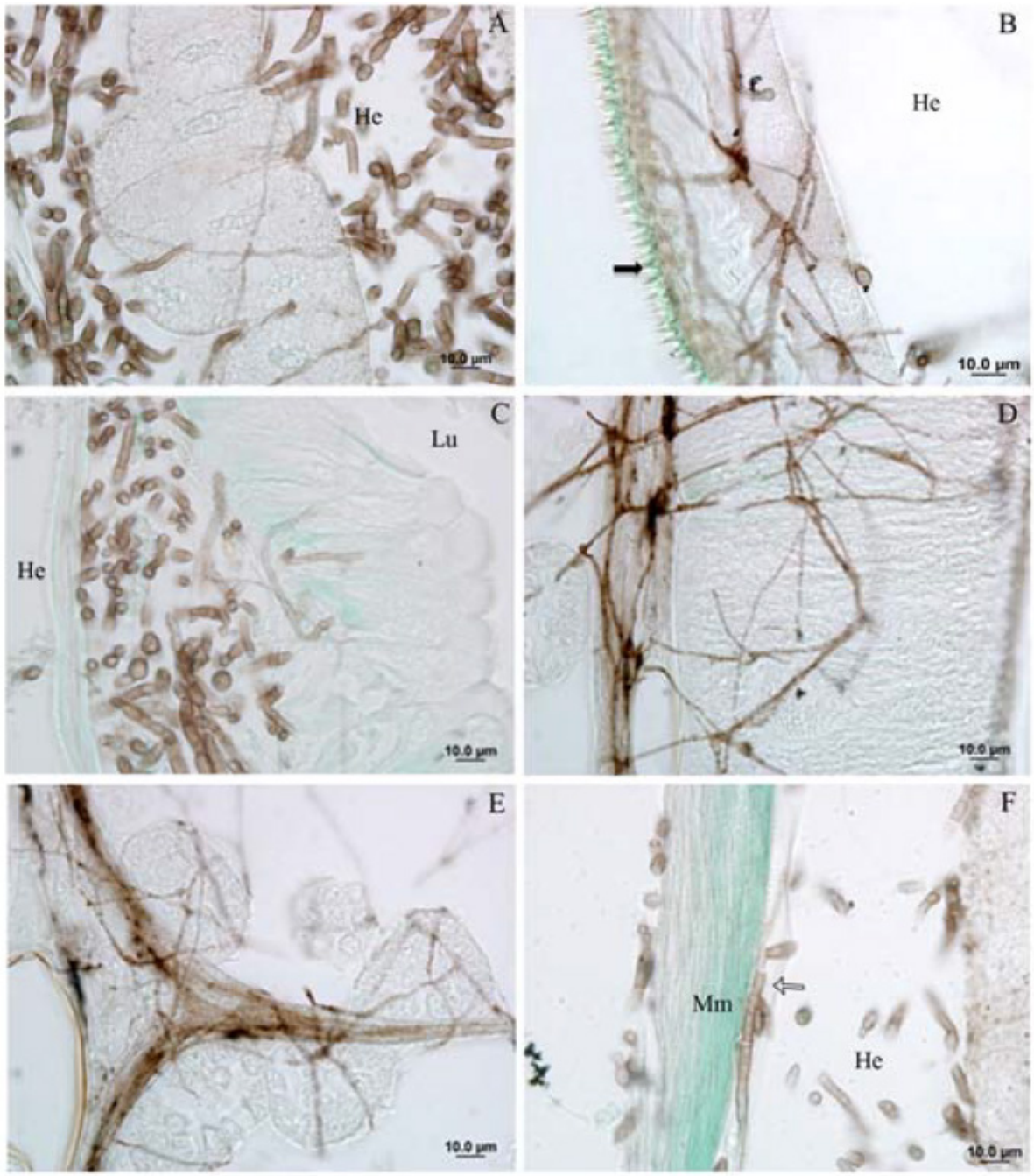

Figure 1 - Photomicrographs of ***B. mori*** tissues infected with ***M. anisopliae***, Gomori staining. Hyphae images, in brown, on fat tissue(A), integument (B), mid gut epithelium (C), silk gland (D), testicle (E), muscle (Mm) and trachea (white arrow) (F). Hemocoel (He), spicules (black arrow), intestinal lumen (Lu).

formation of ***papillae*** on the edge of the epithelial layers and cuticle. As the infectious process advanced from the seventh dpi (Figure 2B) there was separation of the layers and lysis of the epithelial cells, and the area was fully occupied by fungal ***hyphae***.

M. anisopliae intensified growth after the death of insects at the tenth and eleventh dpi, forming a green roof on the integument, which confirmed fungus infection. No other microorganism was observed in the microscopic analysis, probably because the fungus secretes antibiotic substances, which prevent their proliferation (ALVES, 1998). It is noteworthy that although no quantification was performed, it was

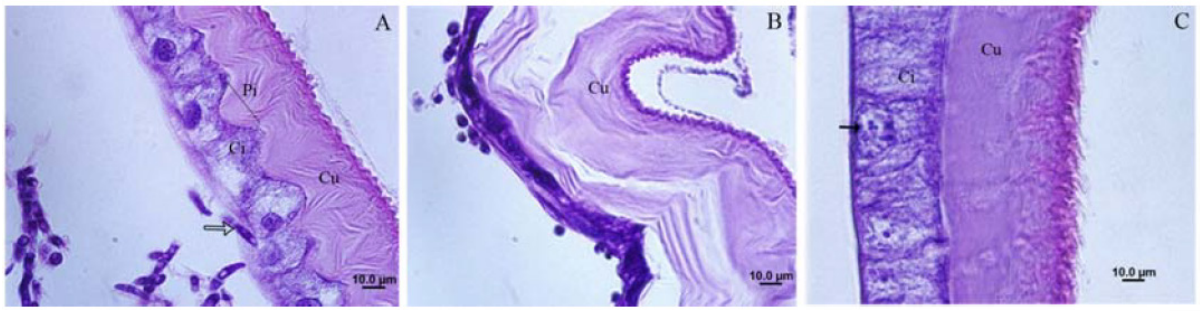

Figure 2 - Photomicrographs integument of ***B. mori*** infected with ***M. anisopliae***. In (A) and (B) changes in integument, in 5th and 8th dpi, respectively. Fungus ***hyphae*** (hollow arrow) and papillae (Pi), visible at the edge of epithelial and cuticular layers. In (B), complete disruption of the integument. (C) Control material for comparison. Cytoplasm (Ci) and nucleus (black arrow) of the integumentary cells epithelium, cuticle (Cu).Hematoxylin and eosin staining.

evident that there was an increase in the number of hemocytes in the hemocoel, on the second dpi, compared to the control, which was also reported by SEWIFY and HASHEM (2001) in relation to ***Galleria mellonella*** (Lepidoptera: ***Pyralidae***). Hemocytes recognise and remove fungal conidia by phagocytosis and encapsulation (CHOUVENC et al., 2009) and on the third dpi it was possible to visualise hemocytes surrounding the hyphal structures. However, this protection is compromised when forming the hyphal bodies, which, after 20 minutes of contact with the hemolymph, express a gene encoding the N-terminal domain of the protein similar to collagen, MCL1. This protein forms a collagenous protective coat in ***hyphae*** and enables ***M. anisopliae*** to evade insect immune responses (WANG &St. LEGER, 2006). Contributing to the infective capacity, ***M. anisopliae*** has a number of protein families, such as ABC transporters, and cytochrome P450, which act in defence against secondary metabolites and detoxification, respectively, and which are produced by the host (GAO et al., 2011). CHOUVENC et al. (2009), SCHRANK & VAINSTEIN (2010) stress the role of toxins, such as destruxins, which weaken the immune defences of the host, cause damage in the muscle system and Malpighian tubules, affect excretion and lead to difficulties in feeding and mobility. This was corroborated in the present study, where a loss in motor coordination was observed between the fourth and fifth dpi.

The combined analysis of symptoms and histopathology revealed that ***B. mori larvae*** are highly susceptible to ***M. anisopliae*** E9 isolate and that death is caused by fungal colonization in the silkworm tissues, resulting in the characteristic symptoms of infection and the mummification of the insect's body.

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