Full Length Research Paper

Infection, colonization and extrusion of *Metarhizium anisopliae* (Metsch) Sorokin (Deuteromycotina: Hyphomycetes) in pupae of *Diatraea saccharalis* F. (Lepidoptera: Crambidae)

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The strains MT and E9 of *Metarhizium anisopliae* were found to be effective in causing the morphological changes in pupae of *Diatraea saccharalis* of different ages. However, during the treatment with the two strains, it was observed that there was a decrease in the longevity of old pupae and adults that emerged from old and young pupae. The adults that emerged died 24 h later and were totally covered by the pathogen's mycelium. Regarding the mortality of pupae of different ages, but in relation to the abnormalities, the pre-pupae were found to be more susceptible. The morphological alterations observed in the cuticle and in the internal tissues of the pupae were analyzed using a stereomicroscope, a light microscope and a scanning electron microscope. The adhesion stage and formation of the appressorium happened 18 h after the infection, occasioning black spots and depressions in the cuticle of young and old pupae. In pre-pupae, the sclerotization was not observed and the fungus acted faster and more effectively, thereby, achieving 100% mortality. The colonization of the pathogen was observed in young pupae between 48 and 120 h. The alterations were observed in the fat body, digestive system and musculature; moreover, several vacuoles were noticed inside the pupae. The extrusion happened after 120 h, presenting the mycelial growth upon the pupae cuticle and further sporulation upon the corpses. The morphological alterations that took place during the development of *M. anisopliae* suggest the possible causes for the mortality of the pupae of *D. saccharalis*.

Key words: Biological control, entomopathogenic fungi, pupal stage, sugarcane borer.

INTRODUCTION

The mycopathogen *Metarhizium anisopliae* (Metsch.) Sorokin and other entomopathogenic fungi are widely known and used as biological control agents for several agricultural plagues of considerable economical expression. This pathogen presents infection and colonization mechanisms similar to those found in the arthropods in their several stages of development and the stages are as follows: adhesion, germination, formation of the appressorium, penetration, extrusion and sporulation upon the corpse (Vicentini and Magalhães,

1996; Alves et al., 2002; Garcia et al., 2005; Bai et al., 2010).

During the colonization, the fungus caused physiological disturbances inside the insect which started on the integument and later reached the circulatory, reproductive, respiratory, nervous, and digestive systems, and interacted with the insect's immune mechanisms; this interaction was very complex and specific (Hajek and Leger, 1994; Hegedus and Khachatourians, 1996; Alves and Pereira, 1998). The penetration of entomopathogenic fungus through the insect's cuticle presents advantages over other pathogens such as virus and bacteria whose infection occurs through oral pathway, mesenteron (Ferron, 1978; Alves, 1998). In the pupal stage, the insect does not eat

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and can be affected by entomopathogenic fungi that penetrate through the cuticle. Several orders of insects such as Diptera, Coleoptera, and Thysanoptera are infected in their pupal stage (Watt and Lebrun, 1984; Ekesi and Maniania, 2000; Destéfano et al., 2005; Ansari et al., 2007); however, the infection and colonization mechanism of the pathogen in this stage has been described a little in Lepidoptera, but has not been demonstrated in larvae (Revathi et al., 2011; Hussein et al., 2012).

The sugarcane borer *Diatraea saccharalis* F. (Lepidoptera: Crambidae) is an insect of great economic interest for the sugar-alcohol industry, because when it occurs in sugarcane plantations, it causes several damages (Roc et al., 1981). Chemical control of the sugarcane borer is not recommended and biological control is the most common method of suppressing *D. saccharalis* (William et al., 1969, Guagliumi, 1973). Biological control of the sugarcane borer larvae by strains of *M. anisopliae* and *Beauveria bassiana* has been achieved (Alves et al., 1984, 1985). In this study, we have evaluated the action of the two strains of *M. anisopliae* in pupae and *D. saccharalis* of different ages, and the morphological alterations in the integument and internal organs during the processes of infection, colonization, and extrusion of the fungus.

MATERIALS AND METHODS

For the acquisition of pre-pupae and pupae, larvae of *D. saccharalis* were placed on artificial diet (Hensley and Hammond, 1968) and kept in a laboratory, under the following conditions: $25 \pm 1^\circ\text{C}$, relative humidity of $70 \pm 10\%$, and 12 h photoperiod. The pre-pupae and pupae of different ages were taken out of the diet and separated into the following groups: pre-pupae (PP, intermediary stage between the caterpillars and the pupae and beginning of the melanization and chitinization), young pupae (YP, from one to six days of development) and old pupae (OP, from 7 to 14 days of development).

Acquisition of the spores

The isolates MT and E9 of *M. anisopliae* were obtained from the micro-organisms Laboratory, ESALQ-USP and was originally isolated from *Deois* sp. (Hemiptera, Cercopidae) in Mato Grosso (MT) and Espirito Santo (E9), Brazil and cultured in Petri dishes containing complete medium (CM) (Pontecorvo et al., 1953), and incubated at $28 \pm 1^\circ\text{C}$, with a relative humidity of $70 \pm 10\%$ for 12 h photoperiod for approximately 10 days. The conidial suspension was prepared by adding 10 ml of an aqueous solution of Tween 80[®] ($0.01\% \text{ v v}^{-1}$) in the dishes containing the isolates. The suspension was removed, filtered in sterilized gauze and the concentration was standardized in 10^7 conidia/ml with the aid of a Neubauer chamber.

Topic treatment

12 individuals were used for each treatment (strain MT, E9, and the control group), using three repetitions ($n = 36$), totalizing 108 individuals per group. Approximately, 100 μl of conidial suspension were sprayed topically on each organism and the control group was

sprayed with the same amount of sterile distilled water. After the treatment, the individuals were all placed into polyethylene containers containing moistened cotton wad, covered with filter paper, and kept under the experimental condition as mentioned above.

A stereomicroscopic analysis

The observations were done in the morning every 24 h until the 9th day after the treatment (216 h) using a Zeiss stereomicroscope (Carl Zeiss, Jena, Germany) and photographed using a TRON 5.2 MG digital camera. The abnormality, mortality, and longevity rates were recorded for further analyses. The emergence and mortality rates of adults of the groups treated were also considered for further analyses.

Statistical analysis

The experimental data of the groups PP, YP and OP were compared applying the analysis of variance (ANOVA). The results that pointed differences between the values were submitted to the test of multiple comparison of Tukey. In all cases, values were considered as significant if the p value was ≤ 0.05 . For these analyses, we used the GraphPad Prism software version 5.0.

Scanning electron microscopic analysis

Young pupae infected with the strain MT of the *M. anisopliae* were fixed in 2.5% glutaraldehyde, 2.5% formaldehyde, and 0.05 M sodium cacodylate buffer, pH 7.2 for 72 h, and post-fixed in 1% osmium tetroxide for 1 h in the same buffer. Samples were dehydrated in a graded series of ethanol and dried under liquid CO₂ (CPD 030; Balzer Union). Specimens were mounted on supports and sputter-coated with a 20 nm layer of gold in the BALTEC (Sputter Coater – SCD 050) and finally investigated under scanning electron microscopy DSM 940A - Zeiss (NAP/MEPA-ESALQ/USP) operating in 20 KV.

Light microscopic analysis

Pupae from the group YP infected with the strain MT of *M. anisopliae* were fixed in the Bouin solution (Junqueira and Junqueira, 1983) on the first day every 6 h after the infection, and after this period, every 12 h until the 9th day (216 h). After being in the fixer for 24 h, the material was subjected to dehydration and inclusion in paraffin according to the routine techniques of histology. Longitudinal cuts of 7 μm of the material were obtained and the sections were stained with haematoxylin-eosin (H/E) and Gomori-Grocott (Behmer et al., 1976). For photo-documentation of slides analyzed with Olympus light microscope, BX-50 was used as a digital 3CCD Pro-series with Image-Pro Plus version 4.5.1 (Media Cybernetics, Sao Paulo, Brazil).

RESULTS AND DISCUSSION

The mortality and abnormality rates in pupae of *D. saccharalis* of different ages treated with the two strains of *M. anisopliae*

As shown in Table 1, the mortality rate does not present any statistical difference among the groups studied with

Table 1. Mortality and abnormality rates (%) of *Diatraea saccharalis* pupae of different ages treated with strains of *Metarhizium anisopliae* under laboratorial conditions.

Group of pupae	Control		Strain MT		Strain E9	
	MOT	AN	MOT	AN	MOT	AN
PP	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	100.0 ± 0.00 ^a	100.0 ± 0.00 ^a	100.0 ± 0.00 ^a	100.0 ± 0.00 ^a
YP	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	83.33 ± 9.62 ^a	55.55 ± 8.01 ^b	77.77 ± 5.55 ^{ab}	48.33 ± 11.67 ^b
OP	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	77.77 ± 5.55 ^a	65.00 ± 5.00 ^b	50.0 ± 9.62 ^b	44.44 ± 5.55 ^b

Mean followed by the standard error. Means that are followed by the same letter in the column do not differ statistically from each other according to Tukey's test ($P \leq 0.05$). PP, Pre-pupae; YP, young pupae; OP, old pupae; MOT, mortality rate; AN, abnormality rate.

the strain MT ($F=3.25$, $df=8$, $p>0.05$) and the abnormality rate is higher in the group PP ($F=18.45$, $df=8$, $p<0.05$). Regarding the strain E9, it presents higher mortality (less significant) and abnormality rates in the group PP ($F=15.25$, $df=8$, $p<0.05$, $F=17.28$, $df=8$, $p<0.05$, respectively). The mortality rate caused by strain MT ranged from 77.8 to 100.00% and that caused by strain E9 ranged from 50.00 to 100.00% among the studied groups, showing that pre-pupae group was the most sensitive for both strains of the fungus (Table 1). It was found that the effects of the infection of *M. anisopliae* in the maturation and reproduction of adults of *Schistocerca gregaria* (Orthoptera: Tettigoniidae) also depended on the age and time of inoculation of the pathogen (Blanford and Thomas, 2001). Adults of *Blattella germanica* (Blattodea: Blattellidae) are more susceptible to isolate ESALQ1037 of *M. anisopliae* infection than nymphs, since mortality of nymphs was always lower than that observed for adults at the same doses (Lopes and Alves, 2011). Ekesi and Maniania (2000) have indicated that the efficiency of *M. anisopliae* at the concentration of 1×10^8 conidia mL^{-1} is higher in adults than in larvae and pupae of *Megalurothrips sjostedti* (Thysanoptera: Thripidae). Thus, it can be mentioned that the susceptibility of insects to pathogenic fungi varies according to the stage of development as well as the tested strain (Ferron, 1985). It was observed that the abnormalities found in pre-pupae and pupae of different ages of *D. saccharalis* were related to the alteration in the integument (Figures 1A, C, F and G). The group PP showed tender cuticle with bright coloration, not presenting any signs of the process of sclerotization (Figure 1A). The other groups, YP and OP presented a totally wrinkled cuticle, with dry aspect and orifices (Figure 1C). This is in contrast to the changes in the larvae and adults of Thysanoptera, which presented an aberrant behavior of the legs and abdomen between three and four days of inoculation of *M. anisopliae* (Vestergaard et al., 1995).

Longevity in young and old pupae of *D. saccharalis* from the emergence of adults obtained from infected pupae with two strains of *M. anisopliae*

By observing the data in Table 2, it appears that the OP

which probably has a short time to finish the metamorphosis from adult pupae has a statistically shorter longevity to the two strains of the pathogen than the control group ($F=8.33$, $df=8$, $p<0.05$); this is different for the group YP that has not presented any difference in any strain in relation to the control ($F=0.62$, $df=8$, $p>0.05$). The ecdysis has been reported as an important factor in the resistance of the insect during the fungus infestation, particularly, when the interval of time between the successive ecdysis is short (Vey and Fargues, 1977). This protective mechanism was proposed by Fargues (1972). After treating *Anastrepha fraterculus* (Diptera: Tephritidae) with *M. anisopliae*, Destéfano et al. (2005) achieved 86% reduction in larval, pre-pupae and pupae populations calculated from the emergence of adults. It was found that the application of *Beauveria bassiana* in the soil was effective in pupae of potato beetle (Coleoptera: Chrysomelidae), where there was a reduction of the emergence of adults from 74 to 77% (Watt and Lebrun, 1984).

Mortality between 40 and 100% are related in pea leafminer, *Liriomyza huidobrensis* (Diptera: Agromyzidae) with isolates of *M. anisopliae* at five days after exposure (Migiro et al., 2010). The mortality of all adults of *D. saccharalis* occurred 24 h after the emergence and this led to 100% mortality that was achieved. After the death, signs of the mycelium growth and the conidiogenesis process were observed upon the corpses evidenced by the white and greenish colorations on the body surface, respectively. Corpses of adult were covered with spores of the fungus 48 h after the insect death (216 h after the infection) (Figure 1 H).

Morphological alterations in the cuticle of the pupae of *D. saccharalis* of different ages during the infection by *M. anisopliae* and extrusion of the pathogen

The development of *M. anisopliae* was observed upon the pupae of *D. saccharalis* of different ages (Figure 1) and the pathogen presented a fast action in the group PP and subsequently, there was mycelium growth upon the integument after 48 h (Figure 1A). Longitudinal cuts in pupae of the group PP revealed that after 72 h, the

Figure 1. Development of MT strain of *Metarhizium anisopliae* on cuticle of pupae of *Diatraea saccharalis* of different ages in a stereomicroscopic analysis. A) PP group covered by the pathogen's mycelium (arrows) at the thoracic region. B) Interior of the integument of PP group evidencing the tracheas (arrows). C) Abnormality in the integument of YP and OP groups, the arrows indicate black spots and depressions in the cuticle. D) The arrow shows the sporulation of the pathogen in the digestive system, YP group. E) The arrow shows large amounts of hyphae pressing the cuticle, YP group. F) Beginning of the extrusion process, show a thorax intersegmental region (arrows), YP group. G) Extrusion process, the arrows show a large amount of hyphae at the intersegmental region (head), spiracles (thorax), and both (abdomen), YP group. H) Corpse of an adult emerged from a YP group. Tr, Trachea; In, intestine; Mi, mycelium. A, B, E, bar, 1 mm; C to D, bar, 2 mm and F, G, H, bar, 3 mm.

Table 2. Longevity (days) of young pupae and old pupae and the emergence rate (%) of adults from young pupae and old pupae of *Diatraea saccharalis* treated with two strains of *Metarhizium anisopliae* under laboratorial conditions.

Treatment	Group of pupae			
	Young pupae		Old pupae	
	Longevity	Emergence	Longevity	Emergence
Control	9.00 ± 0.00 ^a	100.0 ± 0.00 ^a	5.66 ± 0.44 ^a	100.0 ± 0.00 ^a
Strain MT	7.16 ± 0.83 ^a	11.11 ± 5.55 ^b	3.16 ± 0.60 ^b	22.22 ± 5.55 ^b
Strain E9	6.66 ± 1.01 ^a	17.22 ± 9.14 ^b	3.16 ± 0.44 ^b	50.0 ± 9.62 ^b

Values are Mean ± standard error. Means that are followed by the same letter in the column do not differ statistically from each other according to Tukey's test ($P \leq 0.05$).

interior of the pre-pupae appeared empty, showing only the cuticle and tracheas (Figure 1B). It can be seen that the integument of the pre-pupae of *D. saccharalis* is more vulnerable to the penetration of pathogens through instar changes, since the new integument formed was not totally chitinized. The sclerotization propitiates a physical barrier, hardening considerably the integument as well as, a chemical barrier that makes the integument more

stable through the pathogen enzymatic action (Alves and Pereira, 1998). The alteration in the host endocrine balance by *M. anisopliae* was found in adults of *S. gregaria* (Blanford and Thomas, 2001); however, in *D. saccharalis*, the changes observed in relation to the endocrine system were not assessed.

The structure of the cuticle of the young control pupae is shown in Figure 2A. The adhesion, germination of the

Figure 2. Scanning electron microscopic analysis showing alterations in the cuticle of young pupae of *Diatraea saccharalis* during the infection and extrusion processes by the MT strain of *Metarhizium anisopliae*. A) Detail of the surface of the control pupae cuticle, bar: 10 μ m. B) Cuticle infected showing the germinative tube (arrow), bar: 50 μ m. C) Detail of the appressorium forming a site of penetration, bar: 30 μ m. D) The arrows show the orifices in the cuticle, bar: 130 μ m. E) A general view of the thoracic region and abdomen in integument, bar: 300 μ m. F) Detail of the thoracic region, bar: 500 μ m. G) Development of the pathogen's mycelium upon the host's corpse, bar: 10 μ m. H) Formation of conidiophores structures, bar: 50 μ m. I) A corpse totally covered with spores, bar: 50 μ m. Ct, Cuticle.

conidium and the development of the germinative tube on the pupae epicuticle were observed 18 h after the treatment with *M. anisopliae* (Figure 2B). The adhesion occurred probably through the hydrophobic interactions between the conidium and the cuticle of the insect –the production of a layer of adhesive mucus was observed. This extracellular mucilage has been believed to have a role in the support and transportation of enzymes that degrade the cuticle, besides being considered important for the growth of the hyphae and the formation of the appressorium (Zacharuk, 1970; Leger et al., 1991; Bidochka and Leger, 1997; Shahid et al., 2012).

Black spots and several depression sites were noticed in the cuticle of the groups YP and OP 24 h after the treatment with *M. anisopliae* (Figure 1C). These findings probably indicated the degradation of the epicuticle of the

host caused by pathogens which entered through the sites of penetration (Vestergaard et al., 1999; Arruda et al., 2005; Zhang et al., 2010; Yan et al., 2011). The cuticle of the *Galleria mellonella* (Lepidoptera: Pyralidae) larvae infected with *M. anisopliae* and *B. bassiana* became black-spotted due to excessive melanization, indicating direct attack of the fungus on the defense system of the insects (Hussein et al., 2012).

An extensive hyphal growth and a formation of the appressorium with subsequent reaction of melanization in the tissue of the host were found in *Culex quinquefasciatus* (Diptera: Culicidae) (Lacey et al., 1988). A site of penetration from appressorium that was formed by the germination of the conidium 24 h after the infection is shown in Figure 2C. These structures seem to exert a mechanism of pressure on the host cuticle where several

sites of depression are found as observed in the penetration site of the appressorium after 48 h of infection (Figure 2D). The mechanism of pressure may be followed by the enzymatic degradation with the deformation of the cuticle (Figures 2E and F). It was found that the entomopathogenic fungus *M. anisopliae* produces several hydrolytic enzymes such as proteases, chitinases, and lipases in the host's cuticle during the infection process (Leger et al., 1987, 1996; Pinto et al., 1997; Tiago et al., 2002; Krieger et al., 2003; Beys et al., 2005; Shahid et al., 2012). According to Goettel et al. (1989), the penetration on the epicuticle initially happens with the enzymatic degradation and mechanic separation of the lamellae. Thus, it can be mentioned that the mechanism of mechanic pressure and the enzymatic degradation are the probable causes of the abnormalities developed in the integument of pupae of *D. saccharalis* during the infection process of *M. anisopliae* (Figures 1C, 2C, E and F).

In the treatment of *Spodoptera littoralis* (Lepidoptera: Noctuidae), larvae with five strains of entomopathogenic fungi was observed in the cuticle malformations, showing old and dark exuvium. Abnormal pupae attached with moth and fallen moth caused the pupal-moth intermediate stage (Amer et al., 2008). It was found that the colonization occurred between 48 and 120 h when the fungus attacked the interior of the insect before the extrusion (Figures 1D and E). After 96 h of infection, a large amount of mycelium could be noticed, this mechanically pressed the cuticle of the insect to help the emergence of the fungus (Figure 1E). After 120 h of infection, it was seen that the cuticle had breaks from the emergence of the fungus on the surface of the corpse (Figure 1F). Most advanced stage in the extrusion process after 144 h of infection (Figures 1G and 2G). The extrusion process thus, caused great alterations in the host's cuticle probably through physical damages owing to the mycelium growth as well as the beginning of the sporulation process of the fungus (Alves, 1998).

It was observed that the breakage of the insect cuticle and the emergence of the pathogen to the surface (extrusion) began 120 h after the treatment with *M. anisopliae* (Figures 1F and G; 2G, H and I). This process occurs only after the insect death and the hyphae started to emerge from the spiracles using the mechanical pressure through the weakest areas (intersegmental region) and later through the thicker cuticles; thus, the mycelium covers the surface of the whole corpse (Alves, 1998).

The stages of the mycelium of *M. anisopliae* covering the integument of the young pupae and consequently the sporulation upon the corpse between 120 and 192 h after the infection are shown in Figures 2G to I from the beginning of the sporulation upon the corpse to the formation of conidiophore structures 168 h after the infection (Figure 2H). It can be observed from the figure that the hyphae differentiated itself from the corpse

forming several conidia in chains – these structures are denominated as conidiophores (Vicentini and Magalhães, 1996; Vestergaard et al., 1999; Alves et al., 2002; Garcia et al., 2005). A corpse was totally covered with spores of the fungus 192 h after the infection (Figure 2I).

Morphological alterations in the internal tissues of young pupae of *D. saccharalis* during the colonization by *M. anisopliae*

The colonization begins with the penetration process (Figure 2C), which occurs between 24 and 120 h. During this process, the fungus secretes toxins, such as destruxin, which may affect the cells and the host reactions due to the production of *M. anisopliae* (Parry, 1995; Alves, 1998). After 48 h of infection, several vacuoles were found in the body of pupae of *D. saccharalis*. It was noticed in the magnified section that the vacuoles were located inside the fat body and the mycelium of *M. anisopliae* was present in the interiors (Figure 3D). The vacuoles were found in several insects parasitized with entomopathogenic fungi (Vestergaard et al., 1999; Sewify and Hashem, 2001; El-Sinary and Rizk, 2007). It was seen that the colonization of the tissues occurred rapidly in pupae of *D. saccharalis*, presenting the pathogen's mycelium in the fat body of the host in a matter of 60 h (Figure 3A). The process of colonization was also rapid in the hosts, *Frankliniella occidentalis* (Thysanoptera: Thripidae) and *Rhammatocerus schistocercoides* (Orthoptera: Acrididae) treated with *M. anisopliae* and *Metarhizium flavoviride*, respectively (Vicentini and Magalhães, 1996; Vestergaard et al., 1999). Normally, the hyphal growth was found to occur only after the insect death, penetrating in all tissues with filamentous hyphae. The fat body is one of the first organs colonized by the pathogen, which occurs 60 h after of the infection (Figure 3D). It is possible to observe the occurrence of disorganization in this tissue, which according to Alves (1998) affects the metamorphosis of the mature insect, leading to death.

It was seen that the lumen of the alimentary canal was totally occupied by the hyphae 60 h after the infection (Figures 1D and 3D) as described in *B. germanica* (Blattodea: Blattellidae) infected by *Aspergillus flavus* (Pathak and Kulshrestha, 1998). This vegetative growth in the digestive system causes a mechanic blockage in this organ, being one of the reasons for the insect death (Alves, 1998).

It was observed that the mycelium of *M. anisopliae* covers the entire host's body interior in 72 h, forming a mass of hyphae (Figure 3B). In this period, the degradation of the pupa's tissues can be observed, probably by the action of the enzymes released by the fungus. The tissue lysis was also seen by Vestergaard et al. (1999) in the haemocoel of *F. occidentalis*. The formation of structures denominated as chlamydospores

Figure 3. Morphological alterations in the internal tissues of young pupae of *Diatraea saccharalis* during the colonization by the MT strain of *Metarhizium anisopliae* in light microscopic analysis, haematoxylin-eosin (A, B and C) and Gomori-Grocott (D, E and F) staining. A) Detail of the fat body altered by the presence of the fungus. B) Detail of the abdominal region showing a mass of hyphae, the arrow points the structures denominated as chlamydospores, 100 X. C) The arrows indicate the presence of the fungus between the muscle fibres. D) Detail of the vacuoles and intestine with the presence of the hyphae. E) Muscle fibres with hyphae of the pathogens in the interior. F) Muscle fibres altered and separated. Ct, Cuticle; Vc, vacuole; Li, tissue lysis; Mc, musculature; In, intestine. A to C, bar: 160 µm; D, 80 µm; E to F, bar: 160 µm.

in this period as shown in Figure 3B was found in *Galleria mellonella* (Sewify and Hashem, 2001). These structures were also found as the preservation form of the fungus on the ground, possibly preserved inside the corpse (Zacharuk, 1973; Alves, 1998) and were affected by temperature and relative humidity within the eggs of tick (Ment et al., 2010).

The muscle tissue is one of the last tissues of *D. saccharalis* to be colonized by *M. anisopliae*, just before the emergence process, between 60, 72 and 96 h after the inoculation (Figure 3C, E and F, respectively). Deformation can be observed on the structures of muscle fibres, altering their composition as evidenced in the H/E staining (Figure 3C). This mycelial growth is the cause of the lesions in the host's cuticle (Alves, 1998). As shown in Figures 3E and F, inside the muscle fibre, the disorganization of the muscle and the growth of the mycelium in large scale among the musculature and the cuticle can be observed in adult planthopper (Toledo et al., 2010). This is probably due to the fact that the *M. anisopliae*, during its development, caused the separation of the muscle fibres and musculature from the cuticle, facilitating the mechanism of mechanic pressure in the cuticle of the pupae of *D. saccharalis* for its emergence.

The strains MT and E9 of the entomopathogenic fungus *M. anisopliae* are effective for causing mortality and significant abnormalities in pupae of *D. saccharalis* of different ages when compared to the control. The alterations in cuticle and internal tissues such as the digestive system, fat body, and muscle fibres during the infection, colonization and pathogen's emergence processes indicate the host death is an invasion of organs and probably fungal toxin.

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