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Morphological alterations of *Metarhizium anisopliae* during penetration of *Boophilus microplus* ticks

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Abstract. Chronological histological alterations of *Metarhizium anisopliae* during interaction with the cattle tick *Boophilus microplus* were investigated by light and scanning electron microscopy. *M. anisopliae* invades *B. microplus* by a process which involves adhesion of conidia to the cuticle, conidia germination, formation of appressoria and penetration through the cuticle. Twenty-four hours post-infection conidia are adhered and germination starts on the surface of the tick. At this time, the conidia differentiate to form appressoria exerting mechanical pressure and trigger hydrolytic enzyme secretion leading to penetration. Massive penetration is observed 72 h post-inoculation, and after 96 h, the hyphae start to emerge from the cuticle surface to form conidia. The intense invasion of adjacent tissues by hyphae was observed by light microscopy, confirming the ability of *M. anisopliae* to produce significant morphological alterations in the cuticle, and its infective effectiveness in *B. microplus*.

Introduction

Entomopathogenic fungi evolved distinct strategies for their attachment to hosts, varying considerably in their modes of action, virulence and degree of host specificity (Andersen 1979; Alves 1998). Conidial adhesion to host surface and integument penetration are the initial events during the pathogenic process (Askary et al. 1999). Conidia attachment to cuticle is thought to involve non-specific adhesion mechanisms mediated by the hydrophobicity of the conidial cell wall (Boucias et al. 1988, 1991). The attachment is followed by germination and formation of penetration structures from which hyphae are formed in the hemocoel. To transpose the cuticle, the main host barrier, entomopathogenic fungi utilize a combination of mechanical and enzymatic mechanisms. The production of proteases and chitinases is pivotal for penetration by *Metarhizium anisopliae* (Metsch. Sorokin) and other fungi, although other enzymes may also be involved (Boucias and Pendland 1998).

The biocontrol agent *M. anisopliae* is an extensively studied cosmopolitan filamentous fungus, which is a key regulatory organism of insect populations (Inglis et al. 2001; Kassa et al. 2004). In Brazil, *M. anisopliae* is used on a commercial scale for the control of spittlebugs in sugar cane plantations and pastures (Alves 1998). The ability of *M. anisopliae* to infect other arthropods, such as the ticks *Boophilus microplus*, *Ixodes scapularum*, *Rhipicephalus sanguineus* and *Amblyomma maculatum* (Kaaya et al. 1996; Zhioua et al. 1997; Correia et al. 1998; Monteiro et al. 1998a, b; Bittencourt et al. 1999; Frazzon et al. 2000; Kaaya and Hassan 2000; Gindin et al. 2002; Kirkland et al. 2004) and some insect vectors of human pathogens, such as *Culex quinquefasciatus* and *Anopheles gambie s.s.* (Lacey et al. 1988; Scholte et al. 2003), *Triatoma* and *Rhodnius* (Luz et al. 1988), has been reported.

Boophilus microplus is a bovine ectoparasite that causes significant economic losses in tropical and subtropical areas, due to the transmission of diseases that cause reductions in milk yield, and in meat and leather production. The present technology for tick control, which requires significant investment (Castro and Newson 1993), is based on the use of synthetic chemical products. However, the ability of *B. microplus* to develop resistance to acaricides, consumer's demands for chemical free foods, and the negative environmental effects of acaricides call for the development of alternative strategies (Kay and Kamp 1994). Therefore, alternative methods for tick control, such as biological control using filamentous fungi, chiefly, *M. anisopliae* (Correia et al. 1998; Kaaya and Hassan 2000) and *Beauveria bassiana* (Monteiro et al. 1998b), have been pursued.

However, the infection mechanism in ticks, although apparently similar to that reported for insects, is yet poorly described. In this work, we analyze the *M. anisopliae* infection process in *B. microplus* by light and scanning electron microscopy, aiming to identify possible variations, such as infection times and mode of penetration, which may be relevant for tick biocontrol and for the development of commercial formulations.

Materials and methods

Organisms and culture conditions

Metarhizium anisopliae var. *anisopliae* isolate E6 was originally isolated from insects (Rosato et al. 1981) and was maintained as described before (Bogo et al. 1998). Conidia suspensions were prepared from fungi grown on complete Cove's medium (MCc) agar plates (Pinto et al. 1997) in 0.01% Tween 80 solution, washed in sterile distilled water and maintained in 10% glycerol. Prior to use, conidia concentration was determined by direct count using a Neubauer hemocytometer. Suspensions were diluted in sterile distilled water to a final concentration of 1×10^8 conidia ml^{-1}.

Fully engorged females of *B. microplus* were collected from experimentally infested Hereford cattle acquired from a tick free area and housed in individual

tick-proof pens on slatted floors. Cuticles used in the bioassays were obtained from the dissection of some of the ticks under a stereomicroscope.

Bioassays

Groups of 12 ticks were immersed for 30 s in *M. anisopliae* conidial suspensions (10^8 conidia ml^{-1}). Sterile distilled water was applied to control ticks in the same way. After treatment, whole ticks and cuticles were maintained in a humidity chamber (above 90% relative humidity), at 28 °C for 4 days. During this period, ticks were collected daily, examined microscopically for fungal infection, and processed for analysis by light and electron microscopy. Excised tick cuticles were inoculated as described above and prepared for scanning microscopy.

Light microscopy (LM)

Control and infected ticks were fixed overnight at 4 °C with 2% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde solution in 0.1 M sodium cacodylate buffer pH 7.2, dehydrated in 50–95% ethanol and embedded in Leica Historesin. Sections of 3 μm were cut, stained with periodic acid-Schiff (PAS)-green light and examined in a Zeiss MC80 light microscope.

Scanning electron microscopy (SEM)

Whole ticks and cuticles were fixed overnight at 4 °C with 2% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. Post-fixation was carried out in 1% (w/v) osmium tetroxide in the same buffer. The specimens were rinsed in buffer, dehydrated in a series of 30–100% acetone solutions, dried at the critical point in CO_2 (CPD 030 BALTEC) and coated with gold in a sputter-coater (SCD 050 BALTEC). The material was examined in a Jeol JSM 5800 scanning electron microscope (SEM) at the Centro de Microscopia Eletrônica da Universidade Federal do Rio Grande do Sul – CME/UFRGS, Porto Alegre/RS at an accelerating voltage of 20 kV.

Results

Light microscopy

Light microscopy investigation of sections from control ticks clearly identified the three layers of the tick integument: the cuticle, the epidermal cells and the basal membrane (Figure 1a). The cuticle presents three evident layers: endocuticle, exocuticle, and epicuticle, the latter being a thinner layer covering the

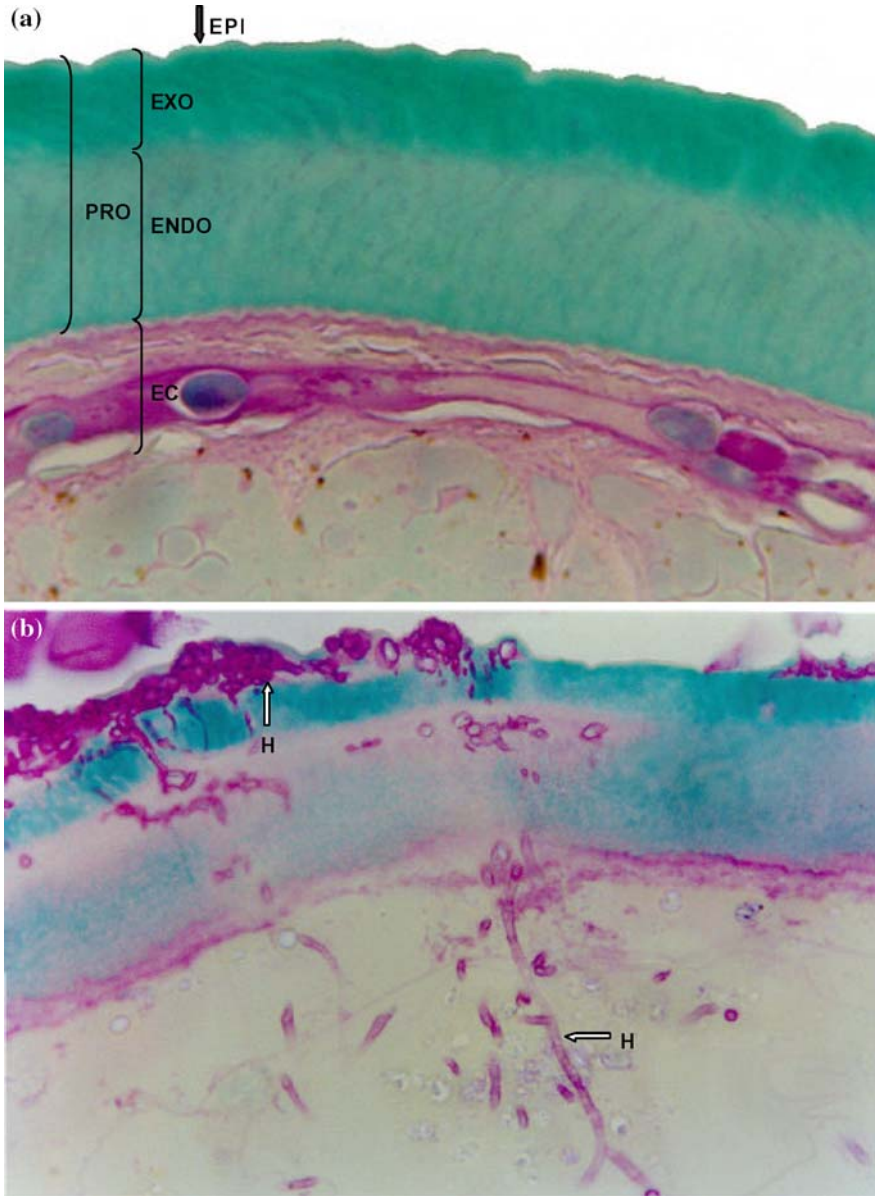

Figure 1. Photomicrography of *Boophilus microplus* integument stained by periodic acid-Schiff. (a) Control cuticle. 640×. (b) PAS reaction of tick *B. microplus* integument infected by *Metarhizium anisopliae*. Note numerous hyphae (H) in the cuticle and adjacent tissue. 640×. EPI: Epicuticle; PRO: Procuticle; EXO: Exocuticle; ENDO: Endocuticle; EC: Epidermal Cells.

former two, which together form the procuticle. In the endocuticle, the polysaccharides were detected by the purple color resultant from the positive reaction to PAS staining. Several epidermal cells are visualized beneath the cuticle (Figure 1a).

The analysis of the colonized *B. microplus* provided an overview of the infection, showing that the invasion of the host cuticle occurs by direct penetration. After forming the penetration structures, the fungus traverses the epicuticle and develops in the transition between the epi- and procuticles (Figure 1b). The hyphae continue their development actively invading the successive procuticle layers, the hemocoel and adjacent tissues, a process which can be visualized in Figure 1b where numerous *M. anisopliae* hyphae can be seen in the adjacent tissues of the tick's cuticle. The fungal cell wall exhibits an intense PAS positive color, due to the presence of polysaccharides, such as chitin, which is evident in the tick cuticle (Figure 1b). Noteworthy is the hyphae dense growth within the procuticle and between it and the epicuticle causing an extensive disorganization of the cuticle layers, with an intense invasion of the body cavity (Figure 1b).

Scanning electron microscopy

Direct penetration of intact cuticle is the normal mode of entry by most entomopathogenic fungi. *M. anisopliae* is not an exception: the conidia are capable of germinating on the host surface and often differentiate to form appressoria. A comparison between the control and colonized ticks is shown in Figure 2. The control ticks (Figure 2a) were preserved after fixation and their surface presented irregular ridges (Figure 2e). In contrast, the ticks inoculated with *M. anisopliae* were wilt with less turgor (Figure 2b) and the infected cuticle showed dense hyphal development 72 h after inoculation. *M. anisopliae* was frequently found forming locally dense hyphal colonies at the cuticle surface (Figures 2c and f).

The SEM analysis of infected ticks showed that *M. anisopliae* conidia are capable of attachment anywhere on the epicuticle surface (Figure 2c), although preferred attachment sites were observed at certain junctions, like those present in the tick legs (Figure 2d). The first sign of conidia germination is the germ-tube extrusion. Each conidium usually produces only one germ-tube (Figure 3), there being a variation in its length prior to appressoria formation: some conidia produced long germ-tubes (Figure 3a), whereas others produced shorter ones (Figure 3b). Globular appressoria were produced at the end of the germ-tubes (Figure 3c), most of them covered by a thin amorphous mucilage layer that firmly adheres the appressoria to the tick integument (Figures 3c, d and e). Sometimes, the germ-tubes penetrated the tick cuticle directly, without any detectable appressoria differentiation (Figure 3f).

At 24 h post-infection, most of the conidia adhered to the host surface (Figure 4a) and the infection process proceeded by germination of the conidia

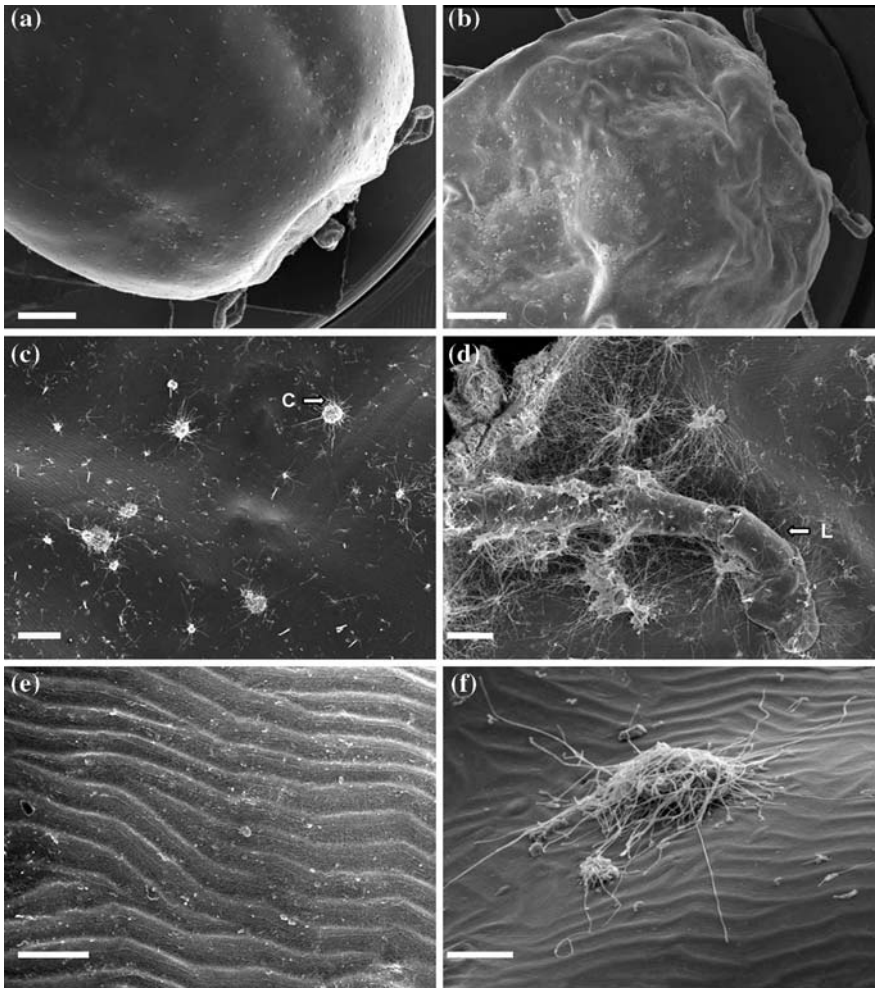

Figure 2. Scanning electron microscopy of the tick *Boophilus microplus*. (a) General view of control tick. Bar = 800 μm. (b) General view of the *Metarhizium anisopliae* infected tick after 72 h post-infection. Bar = 800 μm. (c) and (d) *M. anisopliae* treated cuticle of *B. microplus* after 24 h post-infection. Bar = 200 μm. (e) Detail of the control cuticle surface. Bar = 50 μm. (f) *M. anisopliae* colony in the treated cuticle after 24 h post-infection. Bar = 50 μm. C: Colony; L: Leg.

and by appressoria formation. The appressoria penetrated through the host surface by mechanical (visualized by deformation of the cuticle plane) or enzymatic (visualized by a degradation halo in the host cuticle surrounding the appressoria penetration point) processes (Figures 3b and 4b). At this time, the hyphae were present in the undersurface of host cuticle, as shown in Figure 4c. By 48 h post-infection, most of the conidia were germinated on the tick surface and usually formed long germ-tubes, colonizing the entire host cuticle

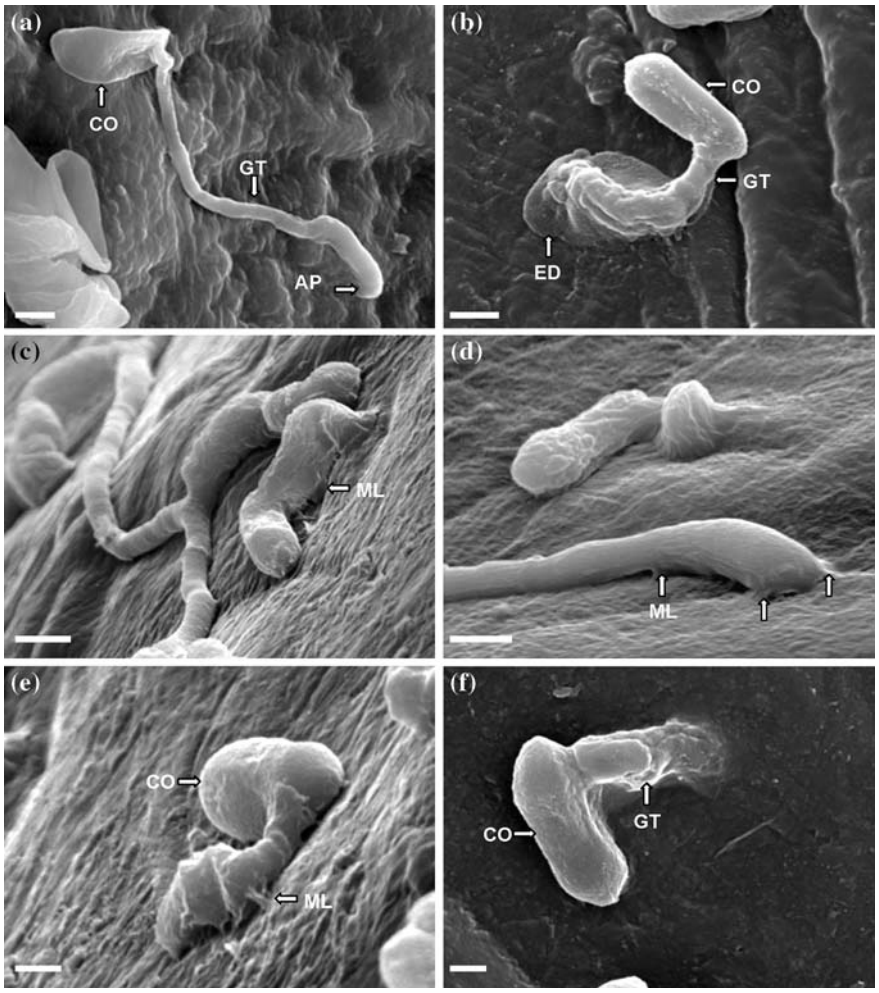

Figure 3. Detail of conidia germination, appressoria formation and penetration of *Metarhizium anisopliae* in the *Boophilus microplus* cuticle. (a) Long germ-tube. Bar = 2 μm. (b) Short germ-tube (GT) and enzymatic degradation (ED). Bar = 2 μm. (c), (d), (e) and (f) Penetration process. (c) and (d) Bar = 2 μm. (e) and (f) Bar = 1 μm. a, b, c, e and f = 72 h post-infection; d = 48 h post-infection. ML: Mucilage Layer; AP: Appressoria; CO: Conidia.

(Figure 4d). Although hyphae penetration starts 24 h post-infection, active penetration was observed between 48 and 72 h after infection. In this period, differences amongst the conidia efficiency to develop penetration structures or local differences in the tick cuticle were clearly observed. During the tick cuticle penetration, the hyphae entered the body cavity and proliferated. Extensive hyphal growth over the cuticle surface is present 96 h post-infection (Figure 4e). At this time, the hyphae started to emerge to the host cuticle

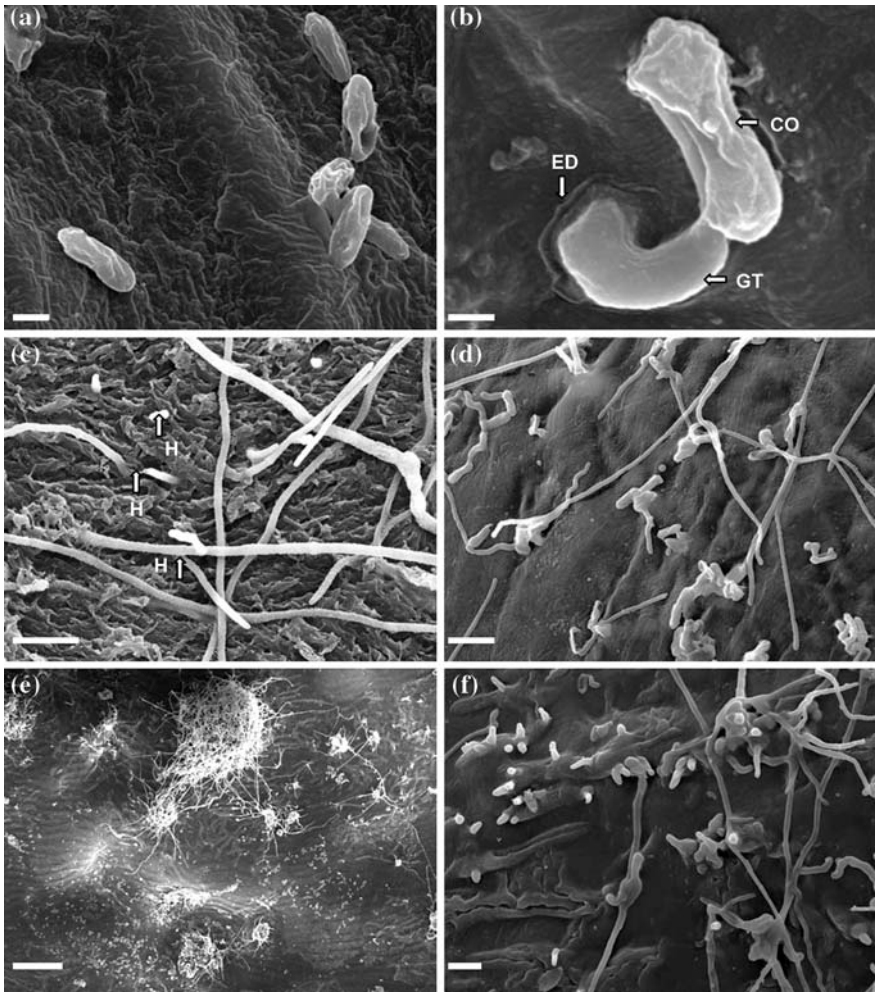

Figure 4. Scanning electron microscopy of *Boophilus microplus* cuticle infected by *Metarhizium anisopliae*. (a) and (b) 24 h post-infection. Bar = 2 and 1 μm, respectively. (c) Undersurface of cuticle showing penetrating hyphae after 24 h post-infection. Bar = 10 μm. (d) Cuticle surface 72 h after infection. Bar = 10 μm. (e) and (f) Cuticle surface after 96 h post-infection. Bar = 100 and 10 μm, respectively. ED: Enzymatic Degradation; CO: Conidia; GT: Germ tube; H: Hyphae.

surface (Figure 4f). Specific points of hyphae emergence were not detectable in the tick cuticle surface.

Evidences of the mechanical pressure exerted by fungal structures are visualized in Figures 5a and b. In several sites a soft depression in the host cuticle is observed at the point of appressoria penetration. The mechanical pressure is accompanied by enzymatic degradation, with cuticle deformation (Figures 3b and 4b) and morphological differentiation of the hyphae (Figure 5c and d).

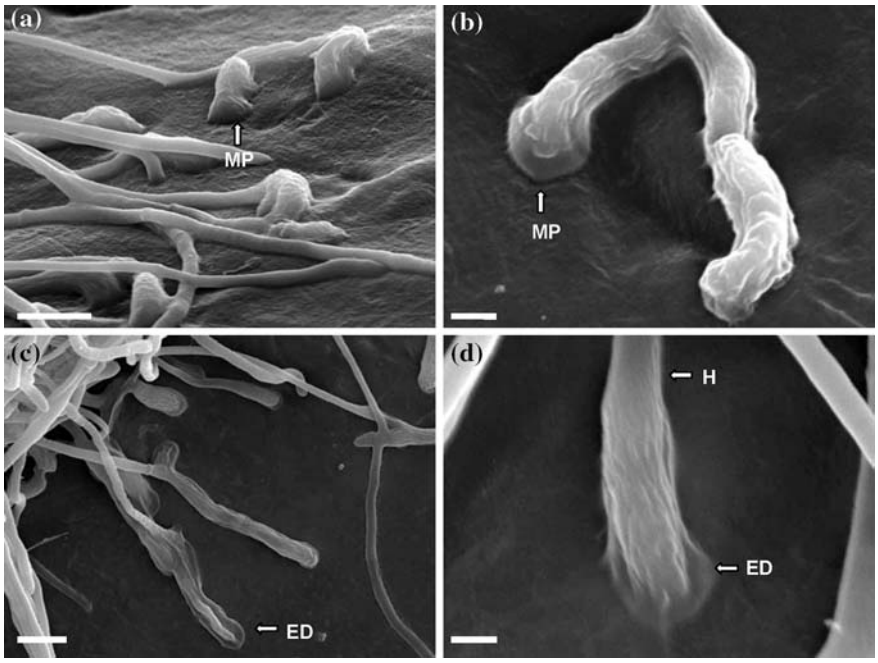

Figure 5. Detail of the direct penetration by mechanical pressure (MP) (a) and (b) and enzymatic degradation (ED) (c) and (d) of *Metarhizium anisopliae* hyphae in *Boophilus microplus* cuticle 48 h post-infection. (a) and (c) Bar = 5 μm. (b) Bar = 2 μm. (d): Bar = 1 μm. H: Hyphae.

These hyphae showed wider extremities when compared to non-penetrating hyphae. Besides that, the cell walls at the tip of penetrating hyphae appear to be less defined. Apparently these morphological alterations are due to enzymes secreted by the penetrating hyphae.

Discussion

In most aspects of the *B. microplus* infection by *M. anisopliae*, our observations are consistent with the commonly described sequence of events that characterizes other entomopathogenic fungal interactions (Alves 1998; Clarkson and Charnley 1996). The infection process involves the following events: (i) conidia adherence to the host cuticle through a thin mucilaginous layer after 24 h post-infection; (ii) conidia germination and development of germ-tube that colonizes the cuticle surface within 24–48 h post-infection; (iii) germ-tube penetration into the tick cuticle within 24–48 h post-infection; (iv) cuticle colonization and emergence of the fungus to cuticular surface within 72–96 h post-infection.

Prior to penetration, conidia need to adhere to the host surface. This is probably mediated by hydrophobic interactions between conidia and the insect cuticle (Bidochka and St Leger 1997) and production of an adhesive mucous layer. The function of the mucoid secretion that surrounds *M. anisopliae* appressorium in *B. microplus* may be similar to that described by other authors in previous work (Zacharuk 1970; St Leger et al. 1987; Bidochka and St Leger 1997; Askary et al. 1999). These authors suggested that the appressoria are enveloped in a mucilaginous secretion and firmly adhere to the host surface over a relatively wide area, furnishing a broad base of attachment. Other studies have shown that mucilaginous subtracts are also produced by the growing hyphal tip and it is not only characteristic of appressoria, but it is also an important feature of hyphal growth and appressoria formation (St Leger et al. 1991). The extracellular mucilage has been proposed to play a role on the support and transport of cuticle-degrading enzymes (St Leger et al. 1996b).

Metarhizium anisopliae conidia readily adhere to the surface of most insects. Our observations revealed that the *M. anisopliae* conidia adhered at any site of *B. microplus*; however, some degree of preference was observed at the tick legs. Similar findings were reported by Vestergaard et al. (1999), which showed that *M. anisopliae* conidia were capable to bind to any site on the cuticle of adult *F. occidentalis*, but were frequently trapped by the setae on the wings and legs. Other studies with *M. anisopliae* (Goettel et al. 1989; Sosa-Gomez et al. 1997) showed that the cuticle regions of *Nezara viridula* contain a large number of setae with high-affinity sites for conidial adhesion. McCauley et al. (1968) reported that infection sites varied within host species and most infections occur in the membranous inter-segmental regions.

Previous work studying *M. anisopliae* infection of Elateridae larvae (McCauley et al. 1968) reported that most conidia germinated within a period of 24–48 h post-infection. Other studies reported that *M. anisopliae* conidia germinated within 12 h post-infection and epicuticle penetration occurred 12–18 h post-infection in *Schistocerca gregaria* (Gunnarson 1988). In *Manduca sexta*, conidia germinated to form appressoria and penetrated the cuticle surface within 40 h post-infection (St Leger et al. 1996a, b; Vestergaard et al. 1999). Vestergaard et al. (1999) studied the infection of *Frankliniella occidentalis* by *M. anisopliae* and reported that conidia germinated within 24 h post-infection, appressoria formation occurred from 30 h post-infection and the colonization was observed after 72 h post-infection. These differences provide evidences that cuticle appears to influence all stages of the infection process and temporal differences in adhesion and germination are important to pathogenicity.

The penetration mode of entomopathogenic fungi is similar to that of plant pathogenic fungi and it is suggested to be based upon a combination of mechanical pressure and enzymatic degradation (St Leger et al. 1987). The entomopathogenic fungus *M. anisopliae* produces a variety of hydrolytic enzymes, such as proteases, chitinases and lipases on the host cuticle during the infection process (Clarkson et al. 1998; Krieger et al. 2003; Pinto et al. 1997; St Leger et al. 1987, 1996a, b; Tiago et al. 2002). The specificity of these enzymes

has important implications in pathogenesis. According to Goettel et al. (1989), the penetration of the epicuticle is primarily by enzymatic degradation, while penetration of procuticle involves both enzymatic degradation and mechanical separation of the lamellae. The supposed enzymatic activity can be visualized by SEM as a clear zone surrounding the penetration point and by the altered morphology observed at the hyphae tips. This alteration at the growing point of the penetrating hyphae has not been reported, although McCauley et al. (1968) have observed, by light microscopy, a clear zone surrounding the penetration peg in insects infected by *M. anisopliae*, and suggested that enzymes diffused from the appressoria.

According to Butt et al. (1995), St Leger et al. (1991) and Vestergaard et al. (1999), differences in the cuticle topography influence the growth form of *M. anisopliae*. In *M. sexta* cuticle (St Leger et al. 1991), appressoria were produced over the hair sockets after extensive growth over the cuticle surface and, in flat surfaces, the appressoria are formed close to the conidia. In *F. occidentalis* short germ-tubes are produced at the end of appressoria in hard parts of the host body, whereas longer germ-tubes are produced at the end of appressoria at membranous wings (Vestergaard et al., 1999). In our study, each conidium usually produced only one germ-tube and the length of the germ-tube is variable, prior to appressoria formation. The appressoria were produced at the end of both long and short germ-tubes, at the same cuticle region. In *B. microplus* cuticles, we found a diversity of germ-tube lengths at the same cuticle location, suggesting that factors other than cuticle topology may influence germ-tube development.

Previous studies revealed that *B. microplus* integument is very similar to those described in insects (Clarkson and Charnley 1996) and in arthropods in general (Wigglesworth 1972). The arthropod's cuticle is the main barrier to *M. anisopliae* infection and protein is the major structural component of insect cuticle (Andersen 1979; Clarkson and Charnley 1996; Clarkson et al. 1998). Our present observations using PAS reaction showed extensive *M. anisopliae* hyphae development within the procuticle and in the transition between pro and epicuticle in the *B. microplus* cuticle post-infection. McCauley et al. (1968) also reported this phenomenon but they observed the simultaneous epicuticle rupture. Clearly, in some of the penetration points, a mass of hyphae completely deforms the cuticle structure, altering its composition, as evidenced by the alteration of its PAS staining.

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