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# Infection and colonization of tissues of the aphid *Myzus persicae* and cassava mealybug *Phenacoccus manihoti* by the fungus *Beauveria bassiana*

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**Abstract** Histopathogenesis of living insects of *Myzus persicae* Sulzer (Hemiptera: Aphididae) and *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae) by *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) was monitored from penetration through insect death. Important events in aphids included fungal penetration of the integument of the less-resistant leg intersegmental membrane and invasion of natural openings, formation of hyphal bodies in live aphids by three days post-inoculation (PI), and extensive hyphal colonization of the two leg segments closest to the insect body at death of the aphids. Confocal microscopy of green fluorescent protein-labeled *B. bassiana* in live mealybugs indicated the fungus penetrated the host through the legs and mouthparts. The fungus was scarce in live mealybugs at 1–5 days PI, formed hyphal bodies by six days PI, and growth was limited to parts of dead hosts at 6–7 days PI. In dead mealybugs, hyphal bodies were near solid tissue. Blastospores were in the hemolymph.

**Keywords** *Beauveria bassiana* (Ascomycota: Hypocreales) · Confocal laser scanning microscopy · Electron microscopy · Entomopathogen · Green fluorescent protein · Insect pathogenesis

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## Introduction

Studies of infection mechanisms and pathogenesis of insects by biocontrol fungi have mainly been concerned with enzymes critical for pathogenesis, invasion of the insect cuticle and initial penetration (e.g., St. Leger et al. 1996a, b; Freimoser et al. 2003; Wang and St. Leger 2005). Nevertheless, six distinct stages in the pathogenesis of insects by *Beauveria bassiana* and *Metarhizium anisopliae* have been detailed: (1) conidial attachment to the insect cuticle, (2) germination and, in some fungi, appressorium formation, (3) invasion and penetration, (4) entry into the hemocoel and host response to fungal invasion, (5) mycosis (disease development inside insects), and (6) external conidiogenesis (Steinhaus 1949; Tanada and Kaya 1993; Kershaw et al. 1999; Bidochka et al. 2000; Charnley 2003; Güerri-Agulló et al. 2010; Toledo et al. 2010).

Although the basic pathogenesis of insects by various fungi follows a similar course, the interaction between a specific insect and fungus varies distinctly. Thus, elucidating specific events will add to our understanding of the underlying mechanisms acting within each organism. Particularly, detailed knowledge on the later stages of pathogenesis within the insect tissues by *B. bassiana* is lacking, for instance, the timing and process of hyphal body formation in the hemocoel, the distribution and density of the fungus within the living host versus the dying/dead host, and the timing and trigger of the fungal switch back to the mycelial phase. Furthermore, previous histological observations of fungal infection of insects are based either on chemically processed tissue or on hemolymph withdrawn from infected insects, so detailed monitoring of events in the natural state of an intact insect is still limited.

*Beauveria bassiana* commonly produces three types of unicellular propagules in culture (Hegedus et al. 1992; Cho et al. 2006): aerial conidia on potato dextrose agar (PDA), submerged conidia in Sabouraud dextrose broth (SDB), and blastospores in SDB with 1 % yeast extract (SDY). On the other hand, protoplast-like cells are formed in insect hosts. These protoplast-like cells include the hyphal bodies that are filamentous-like, elongated or ellipsoidal, and often multicellular and blastospores that are unicellular, usually short, and spherical or oval (Steinhaus 1949; Prasertphon and Tanada 1968; Pendland et al. 1993).

In the present study, we used light, electron, and confocal laser scanning microscopy (CLSM) to investigate in detail infection routes by *B. bassiana* on and below the host cuticle layers and subsequent development in the body cavity of two intact insects, the aphid *Myzus persicae* and the highly destructive cassava mealybug *Phenacoccus manihoti*, which has caused recent cassava losses in Asia of US$ 500 million (Winotai et al. 2010). We chose the small, transparent aphid because cellular events can be clearly observed using light microscopy without destructive preparations. Although the similarly sized mealybug produces a thick layer of wax, which is obstructive to optimal light microscopy, it is more suitable for monitoring living tissues and localizing the fungus as it develops using CLSM because its autofluorescence is much lower than that of the aphid. We therefore used green fluorescent protein (GFP)-labeled *B. bassiana* to view the distribution of the fungus within intact, nonfixed mealybugs at high resolution with CLSM. Here we document events observed over time after the two insect hosts were inoculated with the entomopathogenic fungus *B. bassiana*. Our study should lead to a better understanding of the underlying mechanisms of fungal infection and pathogenesis on insect hosts and thus to better control methods.

## Materials and methods

### Insect rearing and fungal culture

The green peach aphid and the pink cassava mealybug were originally obtained from the Department of Agriculture, Thailand, and subsequently maintained by rearing on kale plants and pumpkins, respectively, in a greenhouse at the BIOTEC Pilot Plant. *Beauveria bassiana* strain BCC2660 was obtained from BIOTEC Culture Collection (BCC). The fungal strain was cultured and maintained on PDA (Difco, USA) at 25 °C. The internal transcribed spacer region of nuclear ribosomal DNA of the fungus was sequenced (GenBank accession No. KC112383), and the sequence confirmed the fungus as *B. bassiana*. Aerial conidia were gently scraped from a 7–10-day-old culture into 10 ml of distilled water, then suspended in 0.1 % Tween 80 (Fluka, UK) and 0.2 % coconut oil (Parisut, Thailand). The concentration was adjusted to $1 \times 10^8$ conidia $ml^{-1}$ for use as inoculum.

### Insect treatments

Two sets of 30–40 green peach aphids or 40 pink mealybugs were treated with a conidial suspension of *B. bassiana* BCC2660 or the GFP-labeled derivative, respectively. Insects were inoculated by either a 10-s dip in the inoculum or with three sprays of the inoculum. Twenty control insects were treated with the same solution without conidia. The inoculated insects were placed on kale leaves (aphids) or pumpkin pieces (mealybugs). The plants or pumpkins were then transferred to plastic boxes and kept in a large carton at 25 °C and 80 % RH. Light and scanning or transmission electron microscopy was used to monitor the infection process of *B. bassiana* on and in the aphid at a minimum of five times: 24, 48, 72, 96, and 120 h post-inoculation (PI). CLSM was used to track fungal infection and distribution in the cassava

mealybug at 24-h intervals for 12 days PI. Three to five aphids or mealybugs were observed at each time.

### Light and electron microscopy

For light microscopy, insects were placed in a drop of 0.05 % cotton blue in lactophenol (Fluka) to stain the fungal cells, then observed with an Olympus CX31 microscope and bright-field optics (Olympus, Japan). Distribution and cell types and shapes of the fungus and the percentage of dead, infected aphids were recorded for each observation period.

Infected and healthy aphids were also observed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). We fixed and prepared samples as described by Hoppert and Holzenburg (1998). Briefly, inoculated aphids were collected at 24, 36, 48, 72 and 96 h PI, then fixed with 2.5 % glutaraldehyde [Electron Microscopy Sciences (EMS), USA] and 2 % paraformaldehyde (EMS) in phosphate buffer (0.1 M $KH_2PO_4$ and $Na_2HPO_4$, pH 7.2), then fixed in 1 % $OsO_4$ (EMS). For SEM, the specimens were dehydrated with an ethanol gradient to 100 % ethanol, then critical-point dried using $CO_2$ (drier model HCP-2; Hitachi, Japan) and sputter-coated with gold–palladium (coater model JFC-1100; Jeol, Japan). Photographs were taken with a SEM (model JSM-35CF; Jeol) at 20 kV. For TEM, the specimens were infiltrated with an epoxy resin (EPON Resin; Epoxy Resins, USA). Sections of 70–85 nm were sliced with a diamond knife (Pelco, USA) and Reichert Ultracut E microtome (Reichert, Austria). The sections were stained with aqueous uranyl acetate (EMS), followed by lead citrate (EMS), and viewed with a TEM (Model JEM-1230; Jeol) at 80-kV accelerating voltage.

### Constitutive expression of synthetic green fluorescent protein gene (sgfp) in *B. bassiana* using *Agrobacterium*-mediated transformation

For constructing a GFP binary vector for fungal transformation, the *bar* cassette (*gpdA* promoter, *bar* coding sequence and *trpC* terminator) was excised from pPZP-Anbar (kind gift from Dr. Charley Christian Staats, Federal University of Rio Grande do Sul, Brazil) with *Eco*RI and *Hin*dIII (Fermentas, Lithuania), then gel-purified and ligated to pUC18 digested with the same restriction enzymes. This recombinant plasmid was called pBar. Then, the *bar*-coding sequence was removed from pBar by digestion with *Nco*I and *Sac*II and replaced with the *sgfp*-coding sequence, which was amplified from plasmid pCT74 (Lorang et al. 2001) using primers sGFP-F-NcoI (5′-GTCAGCCATGGTGAGCAAGGGCGAGGAG-3′; introduced *Nco*I site is underlined) and sGFP-R-SacII (5′-TACGATCCGCGGTCAGCTAGAGGATCCCC TTGTACAGC-3′; introduced *Sac*II site is underlined). The PCR reaction was performed in 50 µl containing 1× Optimized DyNAzyme EXT Buffer (New England BioLabs, USA), 0.2 mM of each dNTP, 1.5 mM Mg, 0.4 mM of each primer, and 1 U of DynaZyme EXT DNA polymerase (New England BioLabs). The thermal cycling program consisted of 5 min at 95 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; and 10 min at 72 °C. The PCR product was gel-purified and ligated to the *Nco*I and *Sac*II sites of pBar as described. This resultant plasmid was named pBar-sGFP. The *gpdA* promoter-*sgfp*-*trpC* terminator cassette was excised from pBar-sGFP with *Eco*RI and *Hin*dIII. The *sgfp* cassette was then treated with DNA polymerase I Klenow fragment (New England BioLabs) to create blunt ends and ligated to *Eco*RI-restricted pPZP-Anbar that was also treated with the Klenow fragment. The resultant binary vector was called pPZP-sGFP-Bar.

Wild-type strain BCC2660 of *B. bassiana* was transformed with pPZP-sGFP-Bar using *Agrobacterium*-mediated transformation (Wang et al. 2010) with some modifications as follows. Two hyphal plugs (6 mm in diameter) were placed in 1 ml water and shaken for 10 s, then 500 µl of this suspension was placed into 50 ml of SDY (Difco). The mixture was shaken at 200 rpm, 28 °C for two days. *Agrobacterium tumefaciens* strain EHA105 was grown in Luria–Bertani broth (Difco) containing 50 µg kanamycin $ml^{-1}$ (A.N.B. Laboratories, Thailand) at 28 °C for two days or until $OD_{660}$ of 0.15. Blastospores (200 µl of $1 \times 10^6$ spores $ml^{-1}$) of *B. bassiana* was mixed with 200 µl of *Agrobacterium tumefaciens* that carried the plasmid pPZP-sGFP-Bar. This mixture was pipetted onto filter paper overlaid on induction medium (de Groot et al. 1998) containing acetosyringone (Sigma-Aldrich, USA). The culture was incubated at 28 °C for two days, then the filter paper was transferred onto minimal medium agar (Epstein et al. 1998) containing 100 µg glufosinate ammonium $ml^{-1}$

(Zhejiang Yongnong Chem, China) and 250 μg cefotaxime $ml^{-1}$ (Siam Pharmaceutical, Thailand). The culture was then incubated at 28 °C for 5–7 days. After two such rounds of culturing for glufosinate-resistance, putative transformants were selected on the same selective medium. Glufosinate-resistant isolates were screened for GFP fluorescence with a Zeiss Axiophot fluorescence microscope (Zeiss, Germany) equipped with an FITC filter set (450–490 nm excitation, 515–565 nm barrier).

### Confocal laser scanning microscopy

CLSM was performed using the FV1000 Fluoview system (Olympus, Japan). All images were recorded at a resolution of 1,024 × 1,024 pixels. All images were taken using a 20×, 40×, or 100× oil immersion objective lens with a 1–3× digital zoom. The image parameters were as follows: laser power: 10–25 %, iris: auto, HV, 400–600, and gain: 1. For each image, the offset voltage was adjusted until the background appeared black or nearly so. Kalman filtering ($n = 4$ or 6) was generally used to improve the signal-to-noise ratio of the images. When the transmitted light channel (TD) was used to capture differential interference contrast images, the argon-ion laser was set at 559 nm. A *z*-series was used to collect images at different specimen depths. For each image, ten sections were collected with focal steps of 1 μm. The images were captured and stored as TIFF files by OLYMPUS FLUOVIEW Ver.3.1a software (Olympus).

## Results

### *Beauveria bassiana* infection of the aphid *Myzus persicae*

The tiny size and the transparent tissue of this aphid facilitated clear, noninvasive observation with light and electron microscopy to document the fungal infection process from 0 to 120 h PI. Immediately after inoculation, fungal conidia were visible on the surface of insect appendages as well as the head, thorax, or abdomen, and by 12 h some had germinated. By 24 h PI, many of the attached conidia had germinated on the cuticle. Rather than penetrating the insect cuticle, germ tubes and hyphae had entered the insect body through natural openings such as the spiracle (Fig. 1a, b), cauda (Fig. 1c), and cornicle (Fig. 1d, e).

In the SEM analysis, conidia had adhered to most parts of the aphid body, especially near the setae on the

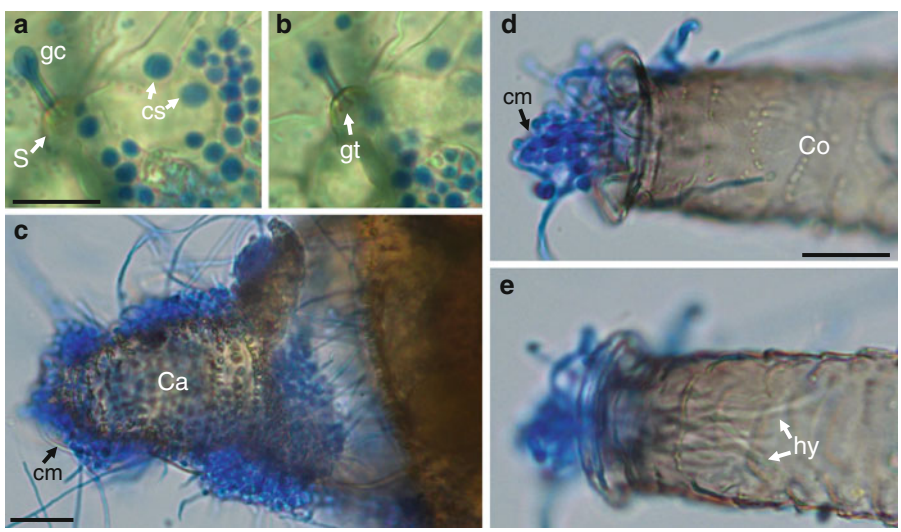


**Fig. 1** Light micrographs of the fungus *Beauveria bassiana* on the aphid host after staining with cotton blue–lactophenol. **a, b** By one day PI, conidia had swelled (cs) or germinated, and germ tubes had grown through a spiracle (S) (seen at different focal planes in **a** and **b**) on a living aphid. Germ tube tip (gt) is marked. Bar 10 μm. **c** At 36 h PI, when some aphids had already died, blue-stained conidial masses (cm) have engulfed the cauda. Bar 10 μm. **d, e** Conidia are present at the opening of the cornicle (Co), shown in surface plane (**d**) and interior plane (**e**), and numerous hyphae (hy) are growing within the cornicle (shown by white arrows). Bar 20 μm

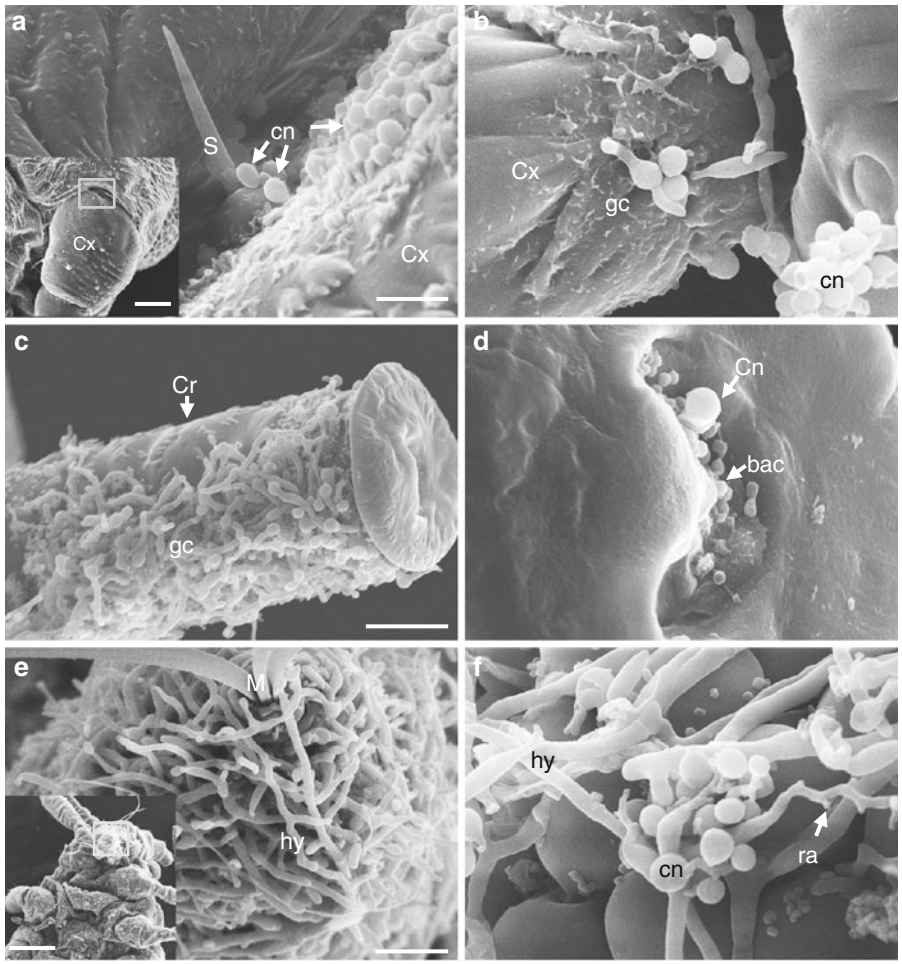


**Fig. 2** SEM of aphid surface at 24–96 h after inoculation (PI) with *Beauveria bassiana*. **a** Conidia (cn) at the socket between the coxa (Cx) and the insect body (indicated by the square in the inset) and at the base of the seta (S). Bar 5 μm as in **b**, **d**, and **f**; inset bar 50 μm. **b** Germinated conidia (gc) around the coxa of the antennae. **c** By 48 h PI, numerous conidia had germinated, and germ tubes had elongated on the side of the cornicle. Bar 15 μm. **d** Conidium on tip of cornicle, apparently surrounded by cocci-shaped bacterial cells (bac). **e** By 72 h PI, hyphae (hy) had colonized and covered the sucking mouthparts (boxed area in inset; bar 150 μm). Bar 10 μm. **f** By 96 h PI, at coxal segments, conidia were present on a zigzag-typed rachis (ra) (arrow)

folded surface (Fig. 2a), which provides crevices suitable for conidial attachment. Conidial germination and hyphal growth was often observed near the intersegmental membrane of the insect legs (Fig. 2b). Hyphae had also entered the host through cornicles (Fig. 2c, d), consistent with our light micrograph data (Fig. 1d, e).

Between 48 and 72 h PI, in living, infected aphids, the fungus was verified to be inside the host body, initially as hyphae, and growing minimally in the hemocoel. We often observed hyphal bodies and blastospores in the hemocoel of legs (Fig. 3). The superficial growth of short germ tubes on aphid legs (indicated by black arrows in Fig. 3a, b) seemed to have been the origin of this development. Colonization at the intersegmental membrane spread to the adjacent leg segments, tarsi and tibiae (Fig. 3b). Within the tibia, hyphal bodies of different sizes and shapes circulated in the leg hemocoel (Fig. 3c–f). Hyphae that grew initially inside the insect body had also fragmented (indicated by arrowhead in Fig. 3d). We actually observed two fungal cells as they floated away from each other (data not shown). Similarly, hyphal bodies were found in a homogenate of aphids that had no sign of external fungal development (data not shown). Also by 48–72 h PI, most inoculated

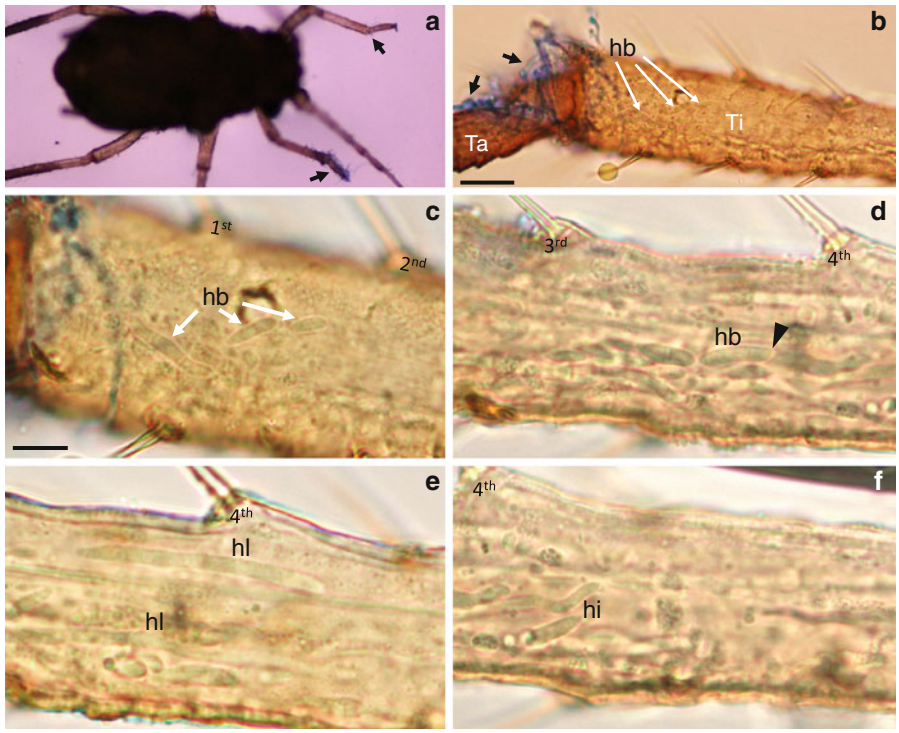


**Fig. 3** Hyphal body formation by *Beauveria bassiana* in the aphid leg hemocoel at 60 h PI viewed with a dissecting microscope (**a**) and a compound light microscope viewed with bright-field optics (**b–f**) with cotton blue–lactophenol staining. **a** The front legs of a living, sick nymph were externally colonized by the fungus at two sites (arrows). **b** Blue-stained, external hyphae (black arrows) in the tarsus (Ta) and numerous hyphal bodies (hb) in the tibia (Ti) near the external infection site of the leg. Bar 5 μm. **c** Close up of three hyphal bodies. **d** In the same leg, hyphae were seen fragmenting into shorter hyphal bodies/blastospores (arrowhead). **e** Two long hyphal bodies (hl). **f** Irregular-shaped hyphal body (hi, black arrow) at the predominant site of infection in this aphid. The first (1st) through fourth (4th) lateral setae of the tibia are shown. Bars are 2 μm in **c–f**

aphids had stopped feeding and spent more time exploring the rearing chamber than the non-inoculated aphids did. Control aphids also continued to feed. In addition, we observed that the infected aphids produced fewer offspring than the non-inoculated aphids did.

TEM analysis indicated that by 72 h after inoculation several internal organs and tissues of some aphids had become heavily colonized by *B. bassiana*. Hyphal bodies and blastospores of various shapes and sizes were detected (Fig. 4a). In some areas, blastospores were near the muscles (Fig. 4b, d) and had penetrated the fat bodies (Fig. 4b), and some hyphae were present near aphid embryos (Fig. 4c). The blastospores that freely float in the hemolymph were mostly spherical or oval and reproduced by budding (Fig. 4e). Interestingly, circulating blastospores in the hemolymph (Fig. 4e) appeared to have thicker, bilayered walls, which were similar to the blastospores produced in SDY (Fig. 4f), but different from those infecting solid tissue (Fig. 4a–d).

By 96–120 h PI, most aphids were dead. Extensive hyphal growth was often observed inside the two leg segments closest to the insect body (coxa and trochanter) (Fig. 5a, b). Likewise, dense mycelia had colonized the interior of many of the dead aphids, from the head to anus (data not shown), even in dead insects that had no external fungal growth. Some severely infected aphids also had advanced paralysis. They were turned upside down or could move only their appendages (data not shown). At or near aphid death, mouthparts were filled with mycelia and conidia (Fig. 2e). Mycelia emerging from aphid cadavers produced aerial conidia on a zigzag-like rachis (Fig. 2f).

### Confocal laser scanning microscopy and distribution of sgfp-expressing *B. bassiana* in pink cassava mealybug *P. manihoti*

Of six transformants obtained after two rounds of selection, isolate GFP3 had the strongest green fluorescence. Isolating a single conidium (Choi et al. 1999), we then generated single-conidial strain of the *sgfp*-expressing *B. bassiana*, GFP3-1 (deposited in BCC, accession BMGC155). Our insect treatments indicated that the wild-type and the *sgfp*-expressing strains similarly caused 50–60 % mortality of the cassava mealybug within seven days. CLSM of mealybugs inoculated with strain GFP3-1 revealed that the distribution of the fungus was similar to the distribution within the infected aphids. However, pathogenesis and insect death was much slower in the mealybugs than in the aphids. At five days PI, most of the inoculated mealybugs were still alive, and few fungal cells were present in the host. The majority of the fungal cells on the host surface were conidia, which had either swelled or produced short germ tubes or had not changed at all (data not shown).

Infected mealybugs had started to die by six days PI. Hyphal bodies, mostly short, 1–2 celled, with tapered ends, had multiplied extensively, notably in

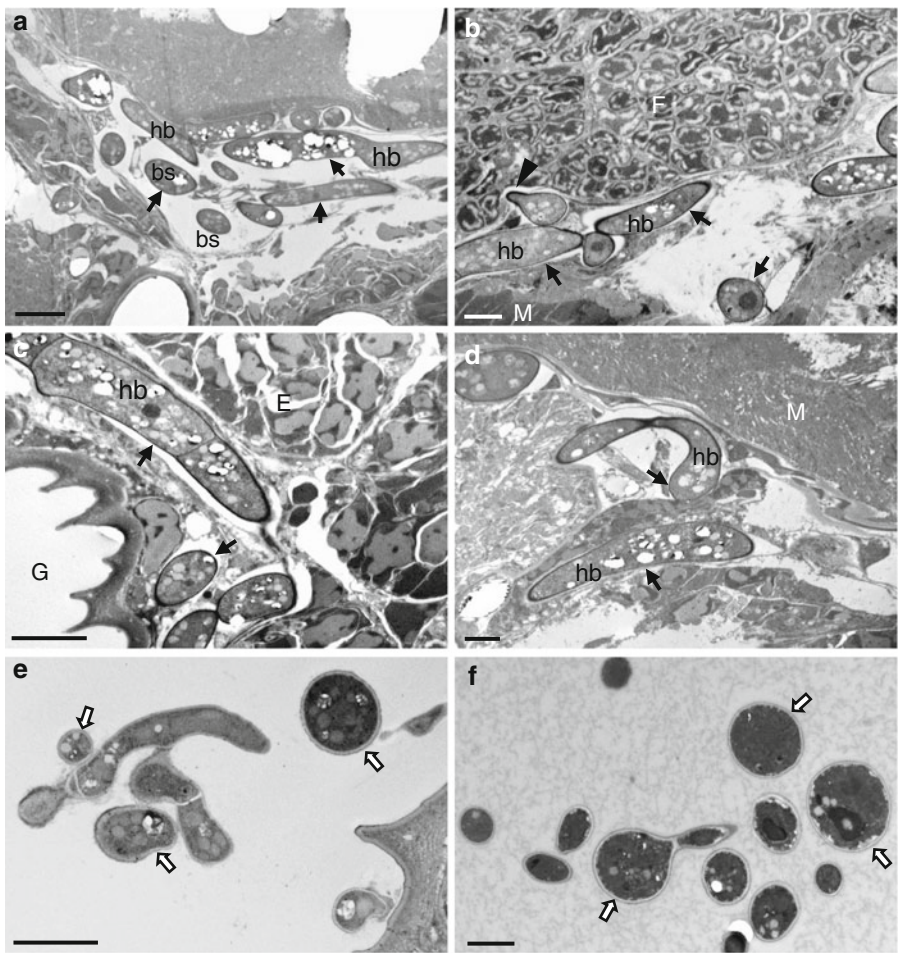


**Fig. 4** TEM of *Beauveria bassiana* cells within severely ill *Myzus persicae* by 72 h PI. **a** Hyphal bodies and blastospores (bs) of various sizes in the hemocoel. Bar 5 μm. **b** Hyphal bodies (hb) have penetrated the fat body (Fb) (black arrowhead). Aphid tissue around the colonized area was degraded. Muscle tissue (M). **c** Hyphal bodies and blastospores penetrated the gut (G) wall and grew near the embryos (E). **d** Fungal cells around muscle (M) of dorsal side of abdomen. **e** Blastospores have grown and branched in the hemolymph. **f** Blastospores had reproduced in SDY broth. Elongated hyphal bodies that were produced in the insect lacked a well-defined cell wall (black arrows in **a–d**), whereas blastospores produced in vivo (**e**) and in vitro in SDY broth (**f**) have a more defined cell wall (white arrows). Bars 2 μm in **b–f**

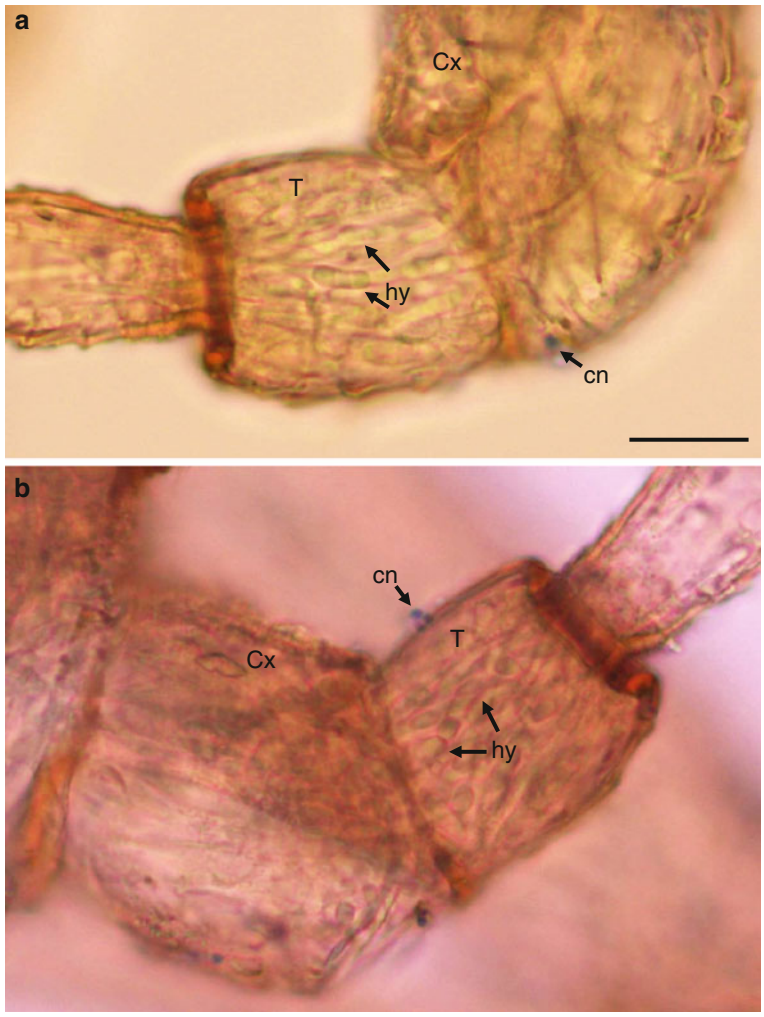


**Fig. 5** **a, b** Light micrographs of trochanter (T) and coxa (Cx) of a leg from two dead aphids filled with hyphae (hy) of *Beauveria bassiana* at 96 h PI. Cotton blue–lactophenol-stained conidia (cn) can be observed on the legs (arrowheads). Bar 10 µm

the leg hemocoel in the coxa, trochanter, and femur segments (Fig. 6a–d). By seven days PI or longer, the hyphal bodies had begun to produce long, thin hyphae (Fig. 6e). In the dead mealybugs, the fungus was limited to certain areas of the host body, for example, the buccal cavity (Fig. 7a, c) and legs (Fig. 7d, e) (where it likely gained entry to the host), the hemolymph (arrows in Fig. 7b), or peripheral solid tissues (arrowheads in Fig. 7b). In the hemolymph, only blastospores were present, whereas hyphal bodies or long hyphae were present on or around the solid tissues and organs (Fig. 7b). At later stages, hyphae grew extensively in the femur of the infected legs of two mealybugs (Fig. 7d, e). Intriguingly, the numerous long hyphal bodies were very uniform in shape and size in the infected femur and oriented parallel with each other and the long axis of the leg (Fig. 7d). At 11 days PI, the fungus grew more rapidly, often forming a hyphal network in leg segments (Fig. 7e) in which the original infection site appeared to have been at the junction between the trochanter and the femur.

## Discussion

Despite the marked difference in the time course of infection in and on the aphid and the cassava mealybug, the basic events during the initial infection and subsequent pathogenesis by the entomopathogenic fungus *B. bassiana* were similar in the two insects. Here, we propose the following model of pathogenesis by the ascomycetous fungus *B. bassiana* on these two insect hosts: (i) The fungus penetrates through the less-sclerotized, less-resistant

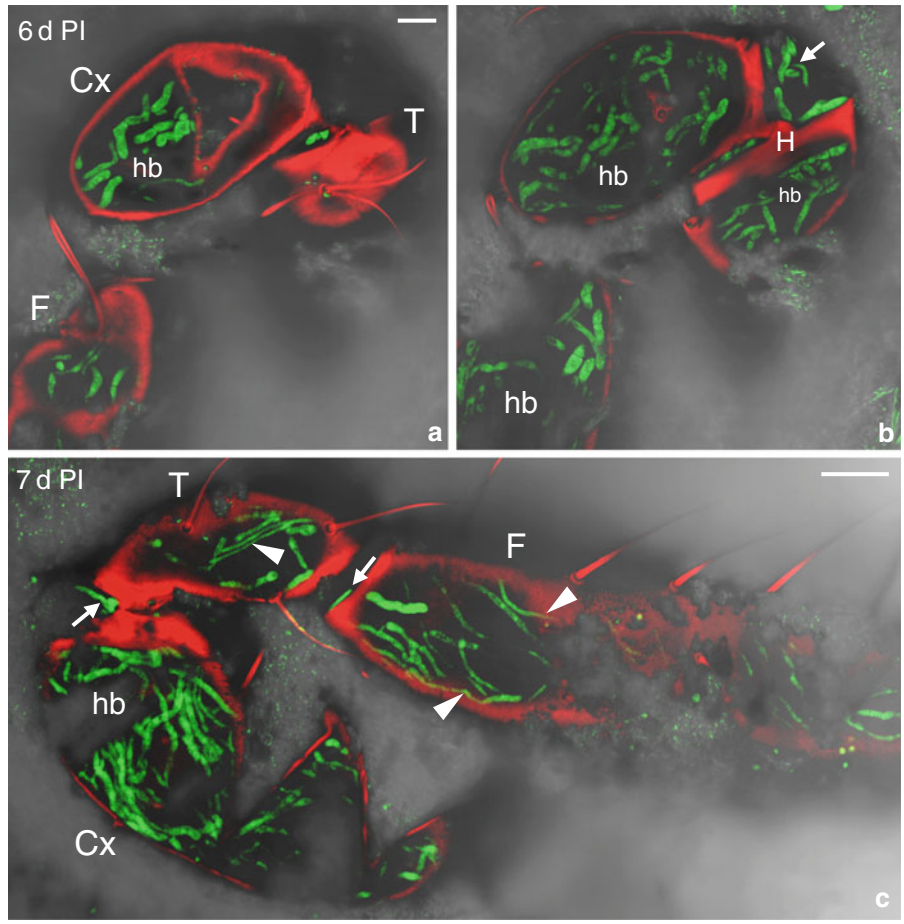


**Fig. 6** CLSM to localize *sgfp*-expressing *Beauveria bassiana* (strain GFP3-1) within leg hemocoel of dead mealybugs. **a, b** Selected optical sections at 1-µm intervals showing green-fluorescing, short, 1–2 celled hyphal bodies (hb) of *B. bassiana* throughout the coxa (Cx), trochanter (T), and femur (F) of leg at six days PI. The fungus is also present at the intersegmental membrane (white arrows, **b, c**). The coxotrochanteral hinge (H) was observed in **b**. Bar 10 µm. **c** By seven days PI, hyphal bodies had begun producing thin hyphae (arrowheads). Bar 20 µm. All images show the green fluorescence from GFP, the red autofluorescence from the insect tissue, and the transmitted light signal

intersegmental membrane of the legs or through natural openings such as the buccal cavity or spiracles. (ii) Once inside the host, a small number of hyphal bodies are produced. (iii) After the initial population of hyphal bodies overcomes any insect defense responses, the number of these protoplast-like cells increases greatly, but fungal growth inside the host is still limited. The infected host may die shortly after that. (iv) *B. bassiana* switches to a mycelial phase; hyphal bodies produce thin, long hyphae, which grow to form an extensive hyphal network in the insect cavity. This phase seems to occur several days earlier in the aphid than in the mealybug.

In the infected aphids, the legs were one of the most susceptible or most frequent sites for fungal infection, perhaps because the legs have a higher density of spines or hair-like structures to anchor conidia. In addition, leg sockets may provide a more favorable microclimate (e.g., higher humidity for germination and growth, shade from UV light) on the host surface because numerous conidia never germinated or had even begun to swell via imbibition on more exposed surfaces of dead insects (data not shown). Germ tubes are also able to penetrate the less-sclerotized, less-resistant skin at the socket. In addition, the lodged fungus may find a rich source of nutrients from the hemolymph if circulation from distal leg segments has become occluded by hyphal growth in the proximal segments. The fungus often colonized the 1st and 2nd segment (coxa and trochanter), perhaps because a

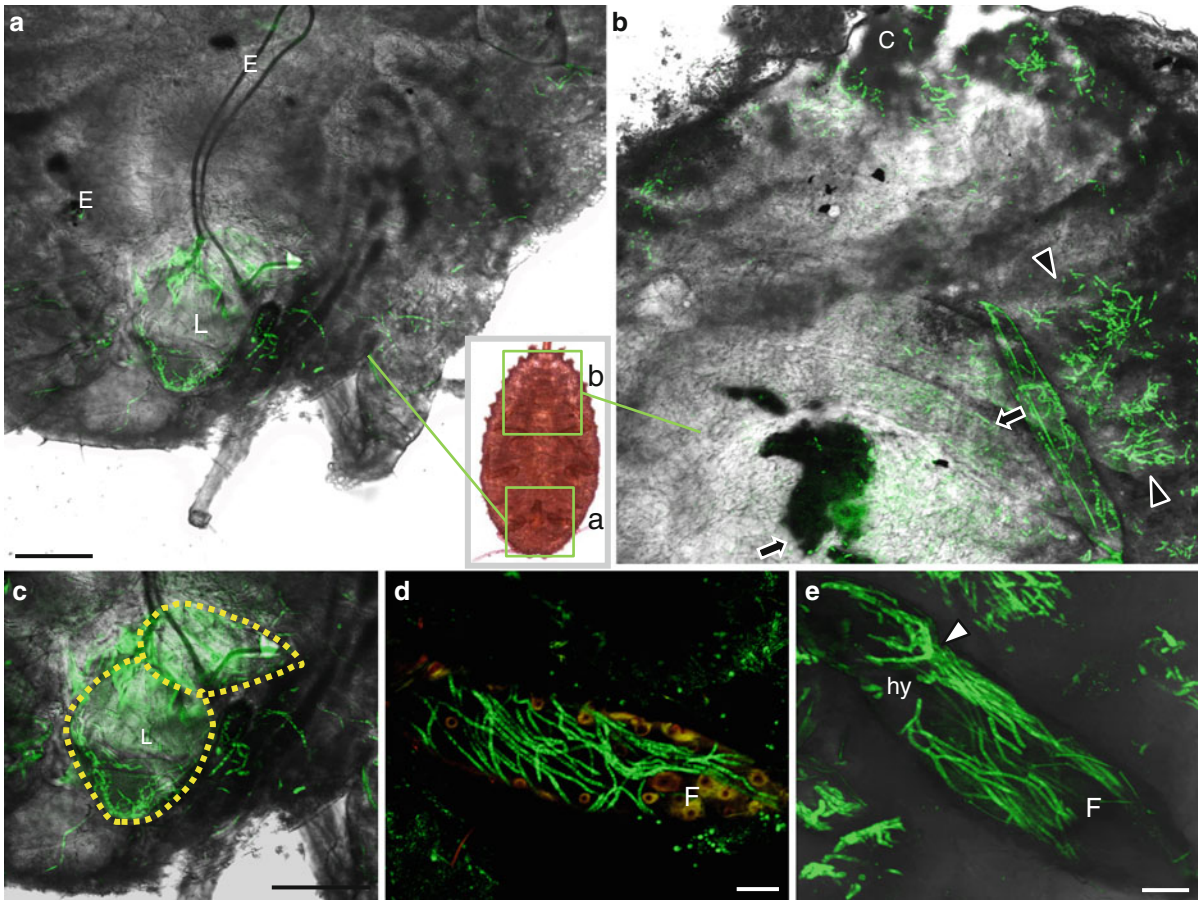


**Fig. 7** CLSM of *sgfp*-expressing strain GFP3-1 of *Beauveria bassiana* within dead cassava mealybugs at seven days PI or longer. **a, b** The fungus was only seen in portions of the host, including the buccal cavity of the labium (L in **a**; insect eyes, E) and leg (in **b**). Unicellular, oval-shaped blastospores were present in the hemolymph (between black arrowheads). Elongated, often, multicellular hyphal bodies were found near the solid tissue (black arrows). Inset ventral side of mealybug showing location of images **a** and **b**. **c** Close up of the labium (L) in **a**, outlined by dotted lines, with numerous hyphal bodies. Bar 80 μm in **a–c**. **d** Femur (F) of insect leg, site of extensive hyphal growth after host death, with long hyphal bodies, similar in size and shape. **e** Hyphal mass (hy) within femur of a different mealybug. Origin of infection was apparently at joint between trochanter and femur (white arrowhead). Bars 20 μm in **e** and **f**

huge gap between the trochanter and the femur (the 3rd segment) provides a substrate for conidial attachment and growth. Confocal micrographs of the *B. bassiana*-infected mealybugs also showed that the fungus grew well in the coxa, trochanter, and femur. CLSM indicated that the fungus grew within the leg intersegmental membrane, which were less- or non-sclerotized. These observations suggest that *B. bassiana* growth progressed along the leg and likely resulted in later colonization of the insect abdomen. The leg of the ectoparasitic mite of honey bees is also the focus of infection by the fungus *Hirsutella thomsonii* (Peng et al. 2002).

The fungus also invaded both insects through natural openings—in aphids, through the cauda, cornicles, mouth-parts, or spiracles, and in mealybugs, the buccal cavity of the two-segmented labium was a site of extensive fungal growth. Our microscopic evidence demonstrated that the fungus apparently spread from the infected labium. The buccal cavity of the sheep blowfly *Lucilia cuprina* is also a site of infection for another entomopathogenic fungus, *M. anisopliae* (Leemon and Jonsson 2012).

Hyphal bodies formed inside the host, a primary event in fungal pathogenesis of an insect host, at approximately 48–72 h PI for *B. bassiana* in the aphid

and apparently originated from the site of external colonization. We also observed hyphal bodies that had been released from the abdomens of living aphids that had symptoms of sickness, and the aphids then started dying quickly. Interestingly, injection of 500 *B. bassiana* blastospores (inoculum) into the leg hemocoel of *Spodoptera exigua* larvae resulted in no increase in hyphal bodies (cells produced in vivo) during the first 24 h PI, but by 36 h PI, dozens of hyphal bodies were present, increasing to 25,000 hyphal bodies $\mu l^{-1}$ hemolymph by 48 h PI (Hung and Boucias 1992). This finding suggests that hyphal bodies that are produced in vivo have a host-adaptive mechanism (e.g., the wall may have been modified to suppress/evade recognition by the host and avoid triggering an immune response) that requires 48 h to develop. Indeed, attachment of *B. bassiana* in vivo hyphal bodies to *Spodoptera exigua* granulocytes occurred at a significantly lower rate than did submerged conidia or in vitro blastospores (Pendland et al. 1993). Because phagocytosis is the property shared between amoebae and insect hemocytes, entomopathogenic fungi were determined for their consequences after internalization by the soil amoeba *Acanthamoeba castellanii*. Unlike *Saccharomyces cerevisiae*, *Penicillium chrysogenum* and *Alternaria alternata* that are killed by the amoeba, *B. bassiana* is able to grow and survive within this amoeboid organism (Bidochka et al. 2010). The long, flexible shape of hyphal bodies could facilitate fungal spread within the host. In contrast, in the cassava mealybug, hyphal bodies were not detected within this host until six days PI, perhaps reflecting greater resistance to *B. bassiana* of the mealybugs in relation to the aphids. In mealybugs that had just died, blastospores/hyphal bodies were only detected in portions of the abdomens. These hyphal bodies then switched to a mycelial phase as they began generating thin hyphal strands. As this necrotrophic phase continued, long hyphal bodies and extensive hyphal networks were detected in the dead insects. Thus, the hyphal bodies appeared to serve as an early infection form of the fungus inside these insect hosts. We are now investigating which genes are expressed in the in vivo-produced blastospores/hyphal bodies and how those genes may be involved in pathogenesis of the insect hosts.

Our TEM evidence suggested that the cell wall of the in vivo-produced hyphal bodies near the peripheral, solid tissue was distinct from the wall of the blastospores produced in vivo in the hemocoel and of those produced in vitro. The fungal wall is composed of two layers: an electron-dense outer layer and an electron-transparent inner layer. The rigidity and strength of the wall, which protects the cell within its environment (e.g., bursting, collapse, osmotic pressure) is conferred by chitin, β(1,3)-glucan, and galactomannan (Griffin 1994). While the blastospores that circulated in the hemolymph or a liquid medium had the well-defined wall structure, hyphal bodies that grew in solid tissue apparently lacked that defined wall. Thus, we hypothesize that in an insect host, synthesis of the hyphal body wall partly depends on the surrounding osmotic potential (whether in a solid or a liquid material). This hypothesis is somewhat in contrast to previous findings that *B. bassiana* hyphal bodies (including those circulating) usually lack a well-defined wall (Pendland et al. 1993). In this previous study, concanavalin A and wheat germ agglutinin were used to demonstrate that galactomannan and chitin, respectively, were missing from hyphal bodies produced in vivo. In a quantitative RT-PCR analysis, a *B. bassiana* chitin synthase gene and a glucan synthase gene were downregulated in in vivo-produced cells, further suggesting modifications in the fungal wall (Tartar et al. 2005).

Because fungal attachment near the most susceptible sites of an insect host, in this case, natural openings and leg intersegments, is a prerequisite for successful pathogenesis by a fungal pathogen, using an attractant trap containing *B. bassiana* conidia may increase the number of conidia contacting suitable infection sites and thus increase control in the field. More research is required to fully determine the viability, longevity and efficacy of fungal conidia in the field.

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## References

Bidochka MJ, Kamp AM, De croos JNA (2000) Insect pathogenic fungi: from genes to populations. In: Kronstad JW (ed) Fungal pathology. Kluwer Academic Publishers, Amsterdam, The Netherlands, pp 171–193

Bidochka MJ, Clark DC, Lewis MW, Keyhani NO (2010) Could insect phagocytic avoidance by entomogenous fungi have evolved via selection against soil amoeboid predators? Microbiology 156:2164–2171

Champlin FR, Grula FA (1979) Noninvolvement of beauvericin in the entomopathogenicity of *Beauveria bassiana*. Appl Environ Microbiol 37:1122–1126

Charnley AK (2003) Fungal pathogens of insects: cuticle degrading enzymes and toxins. Adv Bot Res 40:241–321

Cho EM, Liu L, Farmerie W, Keyhani NO (2006) EST analysis of cDNA libraries from the entomopathogenic fungus *Beauveria* (*Cordyceps*) *bassiana*. I. Evidence for stage-specific gene expression in aerial conidia, in vitro blastospores and submerged conidia. Microbiology 152:2843–2854

Choi YW, Hyde KD, Ho WH (1999) Single spore isolation of fungi. Fungal Divers 3:29–38

de Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat Biotechnol 16:839–842

Epstein L, Lusnak K, Kaur S (1998) Transformation-mediated developmental mutants of *Glomerella graminicola* (*Colletotrichum graminicola*). Fungal Genet Biol 23:189–203

Freimoser FM, Screen S, Bagga S, Hu G, St. Leger RJ (2003) Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. Microbiology 149:239–247

Griffin DH (1994) Fungal physiology, 2nd edn. Wiley-Liss, New York, USA

Güerri-Agulló B, Gómez Vidal S, Asensio L, Barranco P, Lopez-Llorca LV (2010) Infection of the red palm weevil (*Rhynchophorus ferrugineus*) by the entomopathogenic fungus *Beauveria bassiana*: a SEM study. Microsc Res Tech 73:714–725

Hegedus DD, Bidochka MJ, Miranpuri GS, Khachatourians GG (1992) A comparison of the virulence, stability and cell-wall-surface characteristics of three spore types produced by the entomopathogenic fungus *Beauveria bassiana*. Appl Microbiol Biotechnol 36:785–789

Hoppert M, Holzenburg A (1998) Electron microscopy in microbiology. Bios Scientific Publishers Ltd., Oxford, UK

Hung SY, Boucias DG (1992) Influence of *Beauveria bassiana* on the cellular defense response of the beet armyworm, *Spodoptera exigua*. J Invertebr Pathol 60:152–158

Kershaw MJ, Moorhouse ER, Bateman R, Reynolds SE, Charnley AK (1999) The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. J Invertebr Pathol 74:213–223

Leemon DM, Jonsson NN (2012) Comparative studies on the invasion of cattle ticks (*Rhipicephalus* (*Boophilus*) *microplus*) and sheep blowflies (*Lucilia cuprina*) by *Metarhizium anisopliae* (Sorokin). J Invertebr Pathol 109: 248–259

Lorang JM, Tuori RP, Martinez JP, Sawyer TL, Redman RS, Rollins JA, Wolpert JT, Johnson KB, Rodriguez RJ, Dickman MB, Ciuffetti LM (2001) Green fluorescent protein is lighting up fungal biology. Appl Environ Microbiol 67:1987–1994

Pendland JC, Hung SY, Boucias DG (1993) Evasion of host defense by in vivo-produced protoplast-like cells of the insect mycopathogen *Beauveria bassiana*. J Bacteriol 175:5962–5969

Peng CYS, Zhou X, Kaya HK (2002) Virulence and site of infection of the fungus *Hirsutella thompsonii*, to the honeybee ectoparasitic mite *Varroa destructor*. J Invertebr Pathol 81:185–195

Prasertphon S, Tanada Y (1968) The formation and circulation, in *Galleria*, of hyphal bodies of entomophthoraceous fungi. J Invertebr Pathol 11:260–280

St. Leger RJ, Joshi L, Bidochka MJ, Roberts DW (1996a) Construction of an improved mycoinsecticide overexpressing a toxic protease. Proc Natl Acad Sci USA 93:6349–6354

St. Leger RJ, Joshi L, Bidochka MJ, Roberts DW (1996b) Characterization and ultrastructural localization of chitinases *Metarhizium anisopliae*, *M. flavoviride*, and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Appl Environ Microbiol 62:907–912

Steinhaus EA (1949) Fungous infections. In: Steinhaus EA (ed) Principles of insect pathology. McGraw-Hill, New York, USA, pp 318–416

Tanada Y, Kaya HK (1993) Fungal infections. In: Tanada Y, Kaya HK (eds) Insect pathology. Academic Press, San Diego, USA, pp 318–387

Tartar A, Shapiro AM, Scharf DW, Boucias DG (2005) Differential expression of chitin synthase (CHS) and glucan synthase (FKS) genes correlates with the formation of a modified, thinner cell wall in in vivo-produced *Beauveria bassiana* cells. Mycopathologia 160:303–314

Toledo AV, Remes Lenicov AMM, López Lastra CC (2010) Histopathology caused by the entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, in the adult planthopper, *Peregrinus maidis*, a maize virus vector. J Insect Sci 10:1–10

Wang C, St. Leger RJ (2005) Developmental and transcriptional responses to host and nonhost cuticles by the specific locust pathogen *Metarhizium anisopliae* var. *acridum*. Eukaryot Cell 4:937–947

Wang Y, DiGuistini S, Wang TC, Bohlmann J, Breuil C (2010) *Agrobacterium*-meditated gene disruption using split-marker in *Grosmannia clavigera*, a mountain pine beetle associated pathogen. Curr Genet 56:297–307

Winotai A, Goergen G, Tamò M, Neuenschwander P (2010) Cassava mealybug has reached Asia. Biocontrol News Info 31:10N–11N

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