ELECTRON MICROSCOPY OF ENTOMOPATHOGENIC FUNGAL INVASION ON THE CABBAGE-HEART CATERPILLAR *CROCIDOLOMIA BINOTALIS* ZELLER (LEPIDOPTERA: PYRALIDAE)

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ABSTRACT

The infection on *Crocidolomia binotalis* Zeller by the entomopathogenic fungi *Paecilomyces fumosoroseus* (Wise) Brown and Smith, *Beauveria bassiana* (Bals.) Vuill and *Metarhizium anisopliae* var. *majus* (Metsch.) Sorokin was studied histopathologically using electron microscopy. Conidia which landed on the cuticle germinated within four to six hours. In the case of *B. bassiana*, shortly after landing, an appressorium was formed that attached itself fast onto the cuticle at the point of fungal entry. The infected larva then entered the moribund state 12 to 24 hours after inoculation. Death of the larva followed 24 to 48 hours later. By then the body cavity as well as the trachea were clogged with mycelia, and compacted masses of conidiophores producing white conidia were found all over the larval cadaver one to two days later. *P. fumosoroseus* sporulated more abundantly on the surface of the cadaver compared to *B. bassiana* or *M. anisopliae*.

Keywords : *Electron microscopy, entomopathogenic fungi, histopathology*

1. INTRODUCTION

The cabbage-heart caterpillar, *Crocidolomia binotalis* Zeller (Lepidoptera: Pyralidae), has been reported as the second most serious pest of cabbages in the Cameron Highlands of Malaysia (Ooi and Kelderman, 1979). The problem becomes more serious during the drier months and was further aggravated with the intensive use of insecticides without regard for the safety of natural enemies.

A strategy using parasitoids is an integral part of an integrated pest management policy in vegetable farms, but so far none has been reported in Malaysia. In the near future, microbial control may likely play a crucial role in the management policy. Against this

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background, we recently explored the possibility of using indigenous entomopathogenic fungal isolates, *Beauveria bassiana* (Bals.) Vuill, *Metarhizium anisopliae* var. *majus* (Metsch.) Sorokin and *Paecilomyces fumosoroseus* (Wise) Brown and Smith as natural enemies for *Crocidolomia binotalis* Zeller. These fungal isolates have been shown to be infective against the notorious cosmopolitan diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) (Ibrahim and Low, 1993; Ibrahim and Hashim, 1998; Hashim *et al.*, 1999). Their effects on *C. binotalis* have also been established (Hashim and Ibrahim, 2002). At concentration of 2×10^6 conidia mL⁻¹ larval mortality was observed in excess of 80%. Mortality of 100% was recorded at 2×10^7 conidia mL⁻¹ for all the three fungal species. *P. fumosoroseus* was the most efficacious with EC₅₀ at 1.926×10^3 conidia mL⁻¹ and LT₅₀ of less than two days. This study examines the histopathological development of the above entomopathogenic fungi in infected cabbage-heart caterpillars.

2. MATERIALS AND METHODS

2.1 Insect culture

Cabbage-heart caterpillars fed with fresh hybrid cabbage, *Brassica oleracea* var. *capitata*, were maintained in the insectary under a controlled environment of $28 \pm 2^\circ\text{C}$, $80 \pm 10\%$ RH and with equal alternate (12:12 h) light and dark periods. Distilled water and 5% (w/v) honey were provided separately as food for the adults. In the larvae, only active second instar stages were used. To obtain larvae of standardized age, plants with one-day old eggs from oviposition cages were transferred daily into new cages and the eggs were allowed to hatch.

2.2 Fungal isolates

The origin of the fungal isolates is listed in Table 1. They were each passaged through *Plutella xylostella* larvae and then reisolated as single spore isolates, cultured and maintained at room temperature on potato dextrose agar which had been sterilized for 20 min at 121°C at a pressure of 1.05 kg/cm².

Table 1 : Origin of fungal isolates in Malaysia.

Species	Insect host	Plant host	Location
<i>Paecilomyces fumosoroseus</i>	<i>Pteroma pendula</i> (Psychidae; larva & pupa)	<i>Acacia mangium</i>	Selangor
<i>Beauveria bassiana</i>	<i>Glenia celia</i> (Cerambycidae; pupa)	<i>Theobroma cacao</i>	Sabah
<i>Metarhizium anisopliae</i> var. <i>majus</i>	<i>Oryctes rhinoceros</i> (Scarabaeidae; larva)	<i>Elaeis guineensis</i>	Selangor

2.3 Inoculation

Fungal inoculum was prepared from conidia harvested from three week old cultures by scraping the surface of a culture plate that was flooded in distilled water containing 0.05% Tween 80. A Neubauer haemocytometer was used to estimate the conidial concentration and the resulting suspension was standardized to 2×10^7 conidia mL⁻¹. With the aid of a Sigma® hand atomiser, the conidial suspension was sprayed onto cabbage leaf preparations, each averaging 20 cm², and containing 10 second instar larvae. The leaf preparation was kept fresh by wrapping its stalk in wet cotton and aluminium foil. After application, the leaves were covered with clear plastic to maintain 100% R.H. for 24 hours. Thereafter the plastic was removed.

2.4 Whole larval preparation

Four larvae were selected randomly for treatment 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 hours after inoculation. The larvae were exposed to 2% osmium tetroxide vapour for 24 hours. Subsequently, they were freeze dried for another 24 hours. The larvae were then attached on standard aluminium stubs at varying positions and coated with gold using a Polaron sputter-coater. The larvae were observed under a scanning electron microscope (JOEL JSM-6400 SEM® Japan).

2.5 Histopathological observation

Four larvae were removed from the treated leaves at successive intervals of six hours until the appearance of conidiophores on the larval cuticle that occurred 96 hours after inoculation. These larvae were then fixed in aqueous Bouin at room temperature for 24 hours after which they were rinsed in three changes of distilled water to remove the fixative. The larvae were dehydrated in a graded series of ethanol, cleared in methylbenzoate containing 2% celloidin for three days followed by three changes of benzene for 10 min each. Infiltration was done by immersing the samples in two changes of molten paraplast for 30 min each and subsequently left overnight in the oven (60°C).

Embedding of the larvae in molten paraplast was accomplished when a solid bottom layer had formed in the paper mould (1.5 cm³). The embedded larvae were left at room temperature for four days before sectioning began. The processed larvae were sectioned to 10µm using a rotary microtome (Reichert-Jung model 2030), spread on glass coverslips evenly smeared with Mayer's albumin solution and then left to dry (40°C) overnight. The embedding materials from the sections were extracted with xylene, dehydrated in absolute ethanol and air-dried. The prepared coverslip was then attached to the aluminium stub, coated with gold using a Polaron sputter-coater and observed under a scanning electron microscope (JOEL JSM-6400 SEM® Japan).

3. RESULTS AND DISCUSSION

3.1 General observations

In general, the larva began to show symptoms of infection six to twelve hours after inoculation; the larva stopped eating, turned pale and became sluggish. As infection progressed, there was a gradual loss of functional movement and larvae became immobilised and moribund. Body atrophy followed before death two days after treatment. A compact mass of mycelium was observed on the cuticle followed by the emergence of conidiophores three to five days after treatment.

Scanning electron microscopy revealed conidia of the three fungal isolates adhered all over the host cuticle, in masses at the cervical region and intersegmental membranes on both dorsum and venter regions of the abdomen (Figures 1A & B). The conidia of *P. fumosoroseus* varied in size ranging from 3-5 μm (Figure 2) while conidia of *B. bassiana* consisted of compacted globose heads with diameter between 1-2 μm (Figure 3). Conidial size of *M. anisopliae* var. *majus* ranged between 7-9 μm in length and 1.5-2 μm in width (Figure 4).

Figure 1 : Conidia collected along cervical membrane (A) and setal socket (B).

Figure 2 : Conidia of Paecilomyces fumosoroseus.

Figure 3 : Conidia of Beauveria bassiana.

Figure 4 : Conidia of Metarhizium anisopliae var. majus.

Conidial Germination and Cuticular Penetration

The conidia germinated on the cuticle followed by germ tube penetration four to six hours after inoculation (Figures 5A, B & C). Some germ tubes grew on the cuticle surface without penetrating the cuticle, but most tubes penetrated the cuticle close to the conidium. Integumental penetration was not restricted to any particular site on the larvae but especially common at the thinner intersegmental areas of the body wall.

(A)

(B)

Figure 5 : Germinating conidia of *Beauveria bassiana* (A) *Metarhizium anisopliae* var. *majus* (B) and *Paecilomyces fumosoroseus* (C).

Sajap and Kaur (1990) reported that for *M. anisopliae* attacking termites, germ tube penetration through the cuticle occurred within 24 hours after inoculation. Brobyn and Wilding (1977) reported in aphids that germ tube penetration could be delayed up to a week after inoculation.

The elongated germ tube of *B. bassiana* first formed an appressorium which was attached firmly to the cuticle at the point of penetration (Figures 6A & B). Hanel (1982) summarised that an appressorium should be evolutionarily advantageous for penetration, especially in termites where social grooming is common. The appressorium appeared to be a swollen structure of variable size and shape. During the process of penetration the conidium collapsed and shrunk in size, leaving an empty conidial coat (Figures 7A & B).

Appressorium formation was not observed in the infection by *P. fumosoroseus* and *M. anisopliae* var. *majus*. In this case direct enzymatic activity could be playing the initial role prior to fungal invasion followed by mechanical pressure as was first suggested by Zacharuk (1971) in elaterid larva. The lipolytic enzyme, chitinase, could dissolve the chitin layer of the insect cuticle and thus open the way for penetration (Samsinakova *et al.*, 1971). On dark coloured insects light areas indicating possible enzymatic activity around penetration sites were observed (David, 1967). No colour difference was observed to be associated with integumental penetration of *C. binotalis* larvae with the fungi

Figure 6 : Appressorium of Beauveria bassiana with a short (A) and long (B) germ tube.

Figure 7 : Collapsed conidia of *Paecilomyces fumosoroseus* (A) and *Metarhizium anisopliae* var. *majus* (B).

Figure 8 : Germ tubes of *Beauveria bassiana* (A) and *Metarhizium anisopliae* var. *majus* (B) penetrating through spiracular opening.

studied. The hyphae ruptured and virtually destroyed the cuticle before entering the body.

Evidence of hyphal entry through natural openings was numerous with *B. bassiana* and *M. anisopliae* var. *majus* penetrating via spiracular openings (Figures 8A & B) and setal sockets (Figure 9).

Post-penetration in Host Tissue

Between 12 to 24 hours after inoculation some of the infected larvae were already in the moribund state. Symptoms including sluggishness, decreased irritability and partial paralysis were evident in the later stage of infection. Hanel (1982) suggested that the sluggishness could be caused by the disturbance of the interaction of muscles and synapses. Histological studies with elaterid larvae indicated that toxins produced by *M. anisopliae* incited progressive degeneration of tissues with no appreciable swelling or shrinkage of the cells (Zacharuk, 1971). Crisan (1971) also observed that viable conidia could release toxins into the larval gut. Our observations revealed that mycelia were present in the larval body cavity only after larval death (Figure 10). This concurs with the observation by Sajap and Kaur (1990) on termites in which the fungus did not spread into the tissue until after the death of the insect.

Electron microscopic examinations revealed extensive mycelial growth within the tracheal trunks of infected larvae (Figure 11), especially in cases where hyphal penetrations were detected via the spiracular openings. Lacey *et al.* (1988) observed extensive growth of *M. anisopliae* in the siphon of *Culex quinquefasciatus* to the point of blocking air intake and causing suffocation.

By the fifth day after inoculation the hyphae had erupted through the intersegmental membranes and eventually enveloped the whole thorax. Hyphae also emerged through intersegmental membranes of the ventral surface of the abdomen. The fungi spread

Figure 9 : Germ tubes of *Metarhizium anisopliae* var. *majus* penetrating via setal socket.

Figure 10 : Extensive hyphal growth of *Metarhizium anisopliae* var. *majus* in the tracheal trunk.

Figure 11 : Conidia of *Beauveria bassiana* on branched conidiophores on the larval cadaver.

Figure 12 : Conidiophores and conidia of *Metarhizium anisopliae* var. *majus* on the larval cadaver.

rapidly in successive colonisations and became obvious two days later with the tissues and internal organs of the larvae becoming unidentifiable. cursory observations revealed that *P. fumosoroseus* sporulated more abundantly on the surface of the infected larvae compared to *B. bassiana* or *M. anisopliae*. The conidiophores were closely packed with their branches intertwined. Conidial formation was by a simple abstriction of elongated buds which appeared at the distal ends of the branched conidiophores (Figure 12). Other buds formed below the original one and this continued until chains of conidia were formed. Occasionally, *M. anisopliae* was unable to completely dominate its host; the larvae turned black indicative of secondary invasion of saprophytes or bacteria over the host's cuticle. Zacharuk (1971) reported similar observation with *M. anisopliae* on elaterid larvae.

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