International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 11-02-2021, Accepted: 14-03-2021, Published: 23-04-2021
Volume 6, Issue 2, 2021, Page No. 268-272

Histopathological studies on *Spodoptera litura* against entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*

M Raja*, V Vinolia
Centre for Insect Molecular Studies and Environmental Research (CIMSER), P.G & Research Department of Advanced Zoology and Biotechnology, Loyola College, Nungambakkam, Chennai, Tamil Nadu, India

Abstract
The family Cruciferae contains species of great economic importance, providing much of the world's winter vegetables. These include cabbage, broccoli, cauliflower, Brussels sprouts, turnip, radish, rapeseed and mustard. Among the vegetable brassicas in India, cabbage is grown on a large scale accounting to 10% of the world production. Microbial control agents like bacteria (*Bacillus thuringiensis)*, viruses (Nuclear Polyhedrosis Virus) and entomopathogenic fungi (such as *Beauveria*, *Metarhizium* etc.) are potential against lepidopteran larvae. The entomopathogenic fungal formulations are eco-friendly, economic, target-specific and easily biodegradable.
Spodoptera litura was studied histologically in comparison to untreated larvae for the growth and development of *Beauveria bassiana* and *Metarhizium anisopliae.* Germ tube penetration through the insect cuticle was first observed by 2.5 days after inoculation. The germ tubes grew along the endocuticle between the epidermis and the exocuticle laminae and produced lateral branches. Lysis of the endocuticle occurred before hyphae invaded the epidermis. The hyphae penetrated the epidermis and reached the hemocoel within two and a half days after inoculation. After reaching the hemocoel, the invasive hyphae transformed into hyphal bodies that replicated in the hemolymph by budding. Noninvasive hyphal bodies filled the hemocoel in a few days and all tissues became colonized. Nevertheless, these processes did not affect larval feeding activity or digestion and they appeared to be grossly healthy. The noninvasive hyphal bodies converted to invasive mycelia at 5.5 to 6 days after inoculation, completely ramified throughout all larval tissues, penetrated the cuticle and emerged through it. However, the emerging hyphae differed from the hyphae found in the early stages of infection in that they grew perpendicular to the cuticular surface rather than parallel to it. The hyphae emerged from the cuticle of the dead larva and grew allover the surface forming a white myceliam and later formed green mat. Subsequently, cadavers became completely covered by green conidia. The complete developmental cycle of this *M. anisopliae* isolate in *S. litura* lasted approximately 8 to 9 days.

Keywords: *Spodoptera litura*, entomopathogenic fungi, histopathology, infection

Introduction
Entomopathogenic fungi infect their hosts by penetration through the exoskeleton, gaining access to the haemolymph, producing toxins and grow by utilizing nutrients present in the haemocoel and final death of host insect (Ambethgar, 2009) [1].
The responses to mycopathogens within the haemocoel include phagocytosis, encapsulation and nodulation. However, the effect on fungal elements is uncertain. With the arbitrary injection method, Bidochka and Khachatourians (1987) [2] found that haemocytes of the migratory grasshopper, *Melanoplus sanguinipes*, encapsulate viable conidia of *B. bassiana*, however they fail to suppress conidial germination within the nodule. It was suggested that the production of toxins and extracellular proteases by *B. bassiana* could trigger the evadation of encapsulation (Strand and Pech, 1995 and Schmidt *et al.*, 2001) [21, 18].
Insects lack an acquired immune system but have a well-developed innate response. Initial defenses include the physical barriers of the integument or gut, clotting responses by hemolymph, and the production of various cytotoxic molecules at the site of wounding. Foreign entities that pass these barriers and enter the hemocoel must contend with additional cytotoxic molecules as well as an array of different hemocytes. The insect immune system is further subdivided into humoral and cellular defense responses. Humoral defenses include the production of antimicrobial peptides (Meister *et al.*, 2000; Lowenberger, 2001) [12, 11]. Reactive intermediates of oxygen or nitrogen (Bogdan*et al.*, 2000; Vass and Nappi, 2001) [24], and the complex enzymatic cascades that regulate coagulation or melanization of hemolymph (Muta and Iwanaga, 1996; Gillespie *et al.*, 1997) [14]. In contrast, cellular defense refers to hemocyte-mediated immune responses like phagocytosis, nodulation and encapsulation (Strand and Pech, 1995; Schmidt *et al.*, 2001) [21, 18]. The present study was conducted for pathogenicity of entomopathogenic fungi, *B. bassiana* and *M. anisopliae* against *Spodoptera litura* cabbage.

Materials and Methods
Sources of fungal pathogens preparation of concentration
The fungal formulations were obtained from Sun Agro Biosystems Pvt. Ltd., Chennai and five different concentrations of the fungal formulations *viz.* $1x10^{10}$,$1x10^{9}$, $1x10^{8}$, $1x10^{7}$, and $1x10^{6}$ were prepared using sterile water each concentration having different spores.

Haemocytometer spore counts
Spore count was done in a heamocytometer. For most

measurements 10 haemocytometer squares (0.2 by 0.2 mm) were counted per slide, 5 from each side. The spores of the fungi that were studied easily wetted, and suspended in distilled water for counting.

Bioassay

Two fungal formulations *viz.*, *M. anisopliae* (10^{10} conidia/ml) and *B. bassiana* (10^{10} conidia/ml) bought from Sun Agro Bio system Pvt. Ld., Chennai were evaluated against *S. litura*. Uniform sized cabbage leaf pieces were rinsed in sterile water and shade dried and recommended dose of the each fungal formulation 10^9 conidia /ml and control were prepared using sterile water. The shade dried leaf pieces were dipped in respective concentration of the fungal formulation for about 30 seconds and the excess fluid was drained off. The treated leaf pieces were again allowed for shade drying. Each treated leaf piece was kept in separate plastic container and second instar larvae were allowed to feed on the leaf piece @ 10 larvae/container. Pre-starving was avoided to encourage maximum crawling on treated surface and to avoid immediate settlement in a feeding spot. The mouths of the containers were covered with muslin cloth to prevent larval escape. After 2 days all the larvae were transferred to fresh untreated leaves.

Observations on mortality were recorded till pupation. The same procedure was repeated for all the four fungal formulations. The treated larvae were preserved with Formalin Acetic alcohol (FAA) for the histology study and the symptomically visualized fungal pathogen of cadaver also collected and preserved in FAA for histopathology study.

Confirmative test

The fungal treated dead larvae were incubated in a moist chamber for 3-4 days and randomly selected insects were surface sterilized in sodium hypochlorite (1%) for 1-2 minutes and washed in three serial changes of sterile water to eliminate saprobe growth. Larvae were placed on water agar (water + agar), then transferred to PDA media for pure culture. The presence of the fungus was confirmed through microscope examination (Perior*et al.*, 1995).

Histopathology study

The fungal formulation treated spodoptera larvae was sectioned with microtome and stained with Hematoxyline and Eosin **(**Srisukchayakul *et al.*, 2005). Observations were made on spore germination, hyphal growth and pathogenicity in midgut and cross section of whole body.

Results

Histopathology of *S. litura* infected with *B. bassiana*

The growth and development of *B. bassiana* in *S. litura* was studied histologically in comparison to untreated larvae (Plate 1&2). Germ tube penetration through the insect cuticle was first observed by second day after inoculation. It occurred without appressorium formation. Instead, the germ tubes grew along the endocuticle between the epidermis and the exocuticle laminae and produced lateral branches. Lysis of the endocuticle occurred before hyphae invaded the epidermis. The hyphae penetrated the epidermis and reached the hemocoel within two and a half days after inoculation. After reaching the hemocoel, the invasive hyphae transformed into hyphal bodies that replicated in the hemolymph by budding. Noninvasive hyphal bodies filled the hemocoel in a few days and all tissues became colonized. Nevertheless, these processes did not affect larval feeding activity or digestion and they appeared to be grossly healthy. The noninvasive hyphal bodies converted to invasive mycelia at 5.5 to 6 days after inoculation, completely ramified throughout all larval tissues, penetrated the cuticle and emerged through it. However, the emerging hyphae differed from the hyphae found in the early stages of infection in that they grew perpendicular to the cuticular surface rather than parallel to it. The hyphae emerged from the cuticle of the dead larva and grew all over the surface forming a white mycelial mat (Plate 4ab). They usually died with the anterior portion of their body in an elevated position. Newly-formed conidia were visible on conidiophores at 7.5 days after inoculation. Subsequently, cadavers became completely covered by green conidia. The complete developmental cycle of this *B. bassiana* isolate in *S. litura* lasted approximately 8 to 9 days.

Histopathology of *S. litura* infected with *M. anisopliae*

S. litura was studied histologically in comparison to untreated larvae for the growth and development of *M. anisopliae* (Plate 3). Germ tube penetration through the insect cuticle was first observed by 2.5 days after inoculation. The germ tubes grew along the endocuticle between the epidermis and the exocuticle laminae and produced lateral branches. Lysis of the endocuticle occurred before hyphae invaded the epidermis. The hyphae penetrated the epidermis and reached the hemocoel within two and a half days after inoculation. After reaching the hemocoel, the invasive hyphae transformed into hyphal bodies that replicated in the hemolymph by budding. Noninvasive hyphal bodies filled the hemocoel in a few days and all tissues became colonized. Nevertheless, these processes did not affect larval feeding activity or digestion and they appeared to be grossly healthy. The noninvasive hyphal bodies converted to invasive mycelia at 5.5 to 6 days after inoculation, completely ramified throughout all larval tissues, penetrated the cuticle and emerged through it. However, the emerging hyphae (Plate 4ab) differed from the hyphae found in the early stages of infection in that they grew perpendicular to the cuticular surface rather than parallel to it. The hyphae emerged from the cuticle of the dead larva and grew all over the surface forming a white myceliam and later formed green mat. Subsequently, cadavers became completely covered by green conidia. The complete developmental cycle of this *M. anisopliae* isolate in *S. litura* lasted approximately 8 to 9 days.

Discussion

The germination periods for entomopathogenic fungal conidia vary with host. Germination of the conidia on the integument of *S. litura* (46 to 48 h) was similar to that reported for *Heliothiszea* (Mohamed *et al.*, 1978) [13] and on *Spodoptera frugiperda* (Sutton *et al.*, 1981) [22] but slower than that reported for *Pseudoplusia includes* (less than 8 h) (Kish and Allen, 1978) [10] and *Anticarsia gemmatal* (6 to 18 h) (Boucias & Pendland, 1982) [4] These differences in time could be attributed to the variation in the composition of the integument of different larvae.

Germination of *B. bassiana* and *M. anisopliae* conidia was induced by the addition of larval cuticle and yeast extract to minimal medium (El-Sayed*et al.*, 1993). The penetration of

entomopathogenic fungi through the cuticle is sometimes preceded by the formation of an appressorium that firmly attaches to the epicuticle and provides the fulcrum for the mechanical and enzymatic processes that mediate penetration (Deacon, 1997) [6]. However, appressorium formation was not detected in this study, suggesting that penetration by *B. bassiana* and *M. anisopliae* was primarily enzymatic rather than the mechanical processes.

The detection of endocuticular cell lysis prior to the invasion of hyphae into the epidermis was similar to phenomena described by Getzin (1961) [8] and Mohamed *et al.* (1978) [13]. By contrast, Boucias & Pendland (1982) [4] and Thorvilson *et al.* (1985) [23] did not find lysis of the endocuticle. Once the fungal isolate had successfully penetrated into the hemocoel and transformed into hyphal bodies, there was no recognition by hemolymphopsonins or hemocyte (blood cell) surface receptors and no phagocytosis by circulating hemocytes (Pendland*et al.*, 1988). It is possible that this resulted because of similarity between surface components of entomopathogenic and insect cells allowing the hyphal bodies to evade a host immune response (Pendland and Boucias, 1998) [16]

In contrast, hyphal bodies of *Beauveria bassiana*, *Paecilomyces fumosoroseus*, and *Paeciomyces farinosus*are immediately recognized and phagocytized by insect granulocytes and plasmatocytes (Boucias and Pendland, 1991) [5]. The complete developmental cycle of *B. bassiana and M. anisopliae* isolate in *S. litura* lasted ca. 8 to 9 days and closely paralleled the course of its growth in other noctuid larvae.

Plate 1: Infected insect cadaver *S. litura* with different fungal pathogens

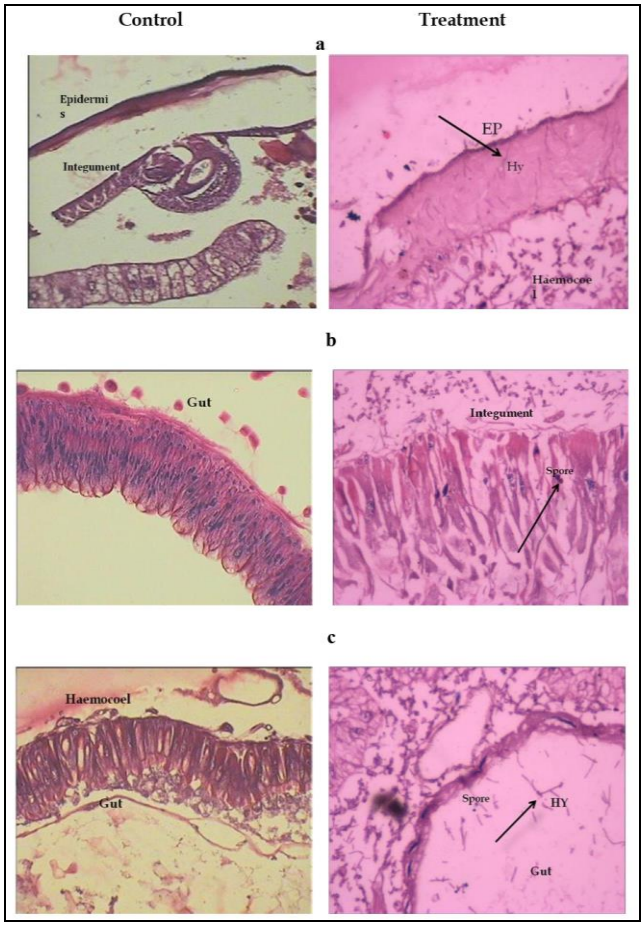

Plate 2: Histopathology of *S. litura* larvae treated with *B. bassiana*

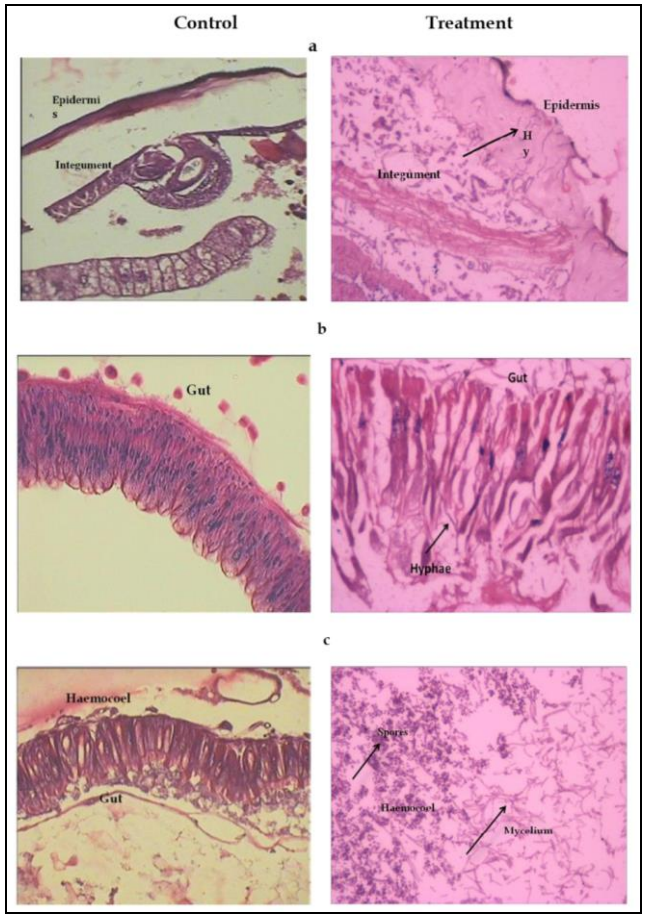

Plate 3: Histopathology of *S. litura* larvae treated with *M. anisopliae*

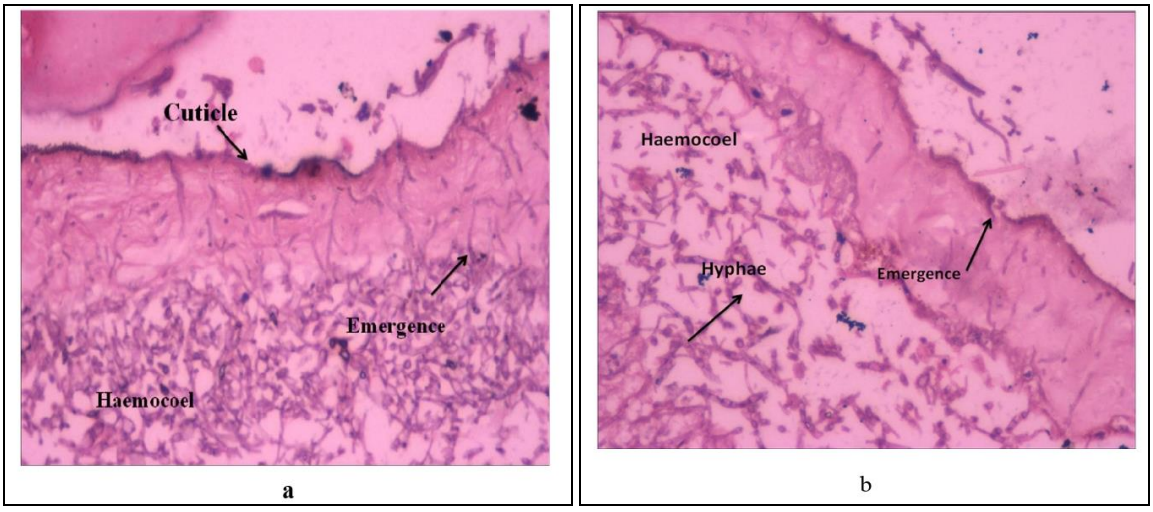

Plate 4: Reemergence of fungal spore

Conclusion

The penetration of entomopathogenic fungi through the cuticle is sometimes preceded by the formation of an appressorium that firmly attaches to the epicuticle and provides the fulcrum for the mechanical. Use of entomopathogenic fungi for *Spodoptera litura* control could have a lot of potential due to their low mammalian toxicity and natural prevalence among larval population. Finally, this study will be useful for the development of *B. bassiaana and M. anisopliae* as a bioinsecticide.

Acknowledgement

The authors are grateful to Loyola College Management for providing the lab facilities to carry out this work. We are also thankful to Dr B. Vasantharaj David for giving more knowledge and valuable suggestions and also Rajiv Ganthi Govt hospital for histopathological work.

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