INTERACTIONS BETWEEN *BEAUVERIA BASSIANA* (BALS.) VUILL. AND THE ENTOMOPATHOGENIC NEMATODES *HETERORHABDITIS BACTERIOPHORA* AND *STEINERNEMA* SP.

III. Histopathological studies of the greater wax moth *Galleria mellonella* L.

By ALIA ZAYED[1] AND MUHAMMAD SHAMSELDEAN[2]

[1]Entomology Dept., Faculty of Science, Cairo University, Giza, Egypt.

[2]Applied Center for Entomonematodes (ACE), Department of Agricultural Zoology and Nematology, Faculty of Agriculture, Cairo University, Giza, Egypt

(Received 27-12-2003)

INTRODUCTION

Penetration of *Beauveria bassiana* (Bals.) Vuill. in its host occurs much more likely through the integument (Ferron, 1985) and possibly through the respiratory organs (Vey and Götz, 1986). The defence mechanism of insects during fungal infection is represented by hemocytic reactions that lead to the formation of capsules, or granuloma whereas phagocytosis does not seem to play an important role (Vey and Fargues, 1977 and Vey and Götz, 1986).

The penetration of the cuticle or epidermal cells by nematodes may elicit a tissue response by the host (Stoffolano and Yin, 1986). Hemocytic encapsulation is involved in the defense mechanism against nematodes which is sometimes accompanied by melanization with or without participation of the hemocytes (Poinar, 1974; Götz *et al.*, 1977).

Interspecific competition from a microbial control standpoint may enhance efficacy by increasing insect susceptibility to nematode infection, reducing the period of lethal infection, or exhibiting a synergistic effect on mortality (Kaya 1993; Koppenhöfer *et al.*, 1999). Continuing the investigations on the dual effect of both entomopathogens, the fungus, *B. bassiana* and the nematodes *Heterorhabditis* *bacteriophora* (isolate EBN 10K) and *Steinernema* sp. (Isolate EGBS), it was imperative to study the pathological effects in the target tissues of the insect.

The combined treatment of greater wax moth *Galleria mellonella* L. last instar larvae by both entomopathogenic fungus and nematodes did not favour the coproduction of both pathogens in the same cadaver as reported by Barbercheck and Kaya (1990) and Zayed *et al.*, (2003). Though *Steinernema* sp. developed almost normally after being incubated *in vitro* with the fungus (Shamseldean *et al.*, 2003), the same nematode failed to reproduce in the cadavers of *G. mellonella* (Zayed *et al.*, 2003). The present work is an attempt to detect how both pathogens would interact together inside the infected last instar *G. mellonella* and to elucidate the histopathological changes occurred due to the infection.

MATERIAL AND METHODS

The colony of *Galleria mellonella* L. was maintained in the laboratory at 23 ± 2°C on sterile bee wax, at the Applied Center of Entomonematodes (ACE), Faculty of Agriculture, Cairo University.

Beauveria bassiana (Bb2) was obtained originally from *G. mellonella* larvae collected from Ismailia Governorate, Egypt and its pathogenicity was evaluated against *G. mellonella* L. larvae. (Zayed, 2003). The fungus was grown on Sabouraud dextrose yeast agar containing, 1% peptone, 0.2% yeast extract, 4% dextrose and 1.5 % agar in distilled water at 23 ± 2 °C for at least 2 weeks. Conidiospores were harvested and suspended in sterile distilled water containing 0.05% Tween 80 from 14 days-old cultures under sterile conditions. The conidial suspensions were adjusted to the desired spore counts using Neubauer haemocytometer.

The entomopathogenic nematodes *Heterorhabditis bacteriophora* (isolate EBN 10K) and *Steinernema* sp. (Isolate EGBS) were isolated from soil samples in El- Nubaria, Beheira and El-Badrashane, Giza, respectively (Atwa, 1999).

The nematodes were cultured in the last instar larvae of *G. mellonella* according to the method of Woodering and Kaya (1988). The infective juveniles (IJs) of both nematodes were harvested from nematode White traps as described by White (1927) at 25 ± 1°C. A stock suspension of the IJs in sterile distilled water was stored at 10 °C for 2 weeks until used. The nematode suspensions were prepared in sterile distilled water at a concentration of 500 IJs/ml from each nematode species.

Groups of twenty last instar larvae of *G. mellonella* each, were treated by either 500 IJs/ml of each nematode species, or a combination between equal volumes of 0.625 spores /ml of *B. bassiana* and the previous nematode concentration from each species. In all treatments larvae were exposed for 24, 48 and 72 hours to 2 ml of either fungal, nematode, or fungus-nematode suspension. Twenty last instar control larvae of *G. mellonella* were exposed to 2 ml sterile distilled water.

Histological preparations:

Survived *G. mellonella* larvae were fixed in alcoholic Bouin's solution for 24 h, then washed and dehydrated in an ascending series (70-100%) of ethyl alcohol. Infiltration of samples took place according to Gad (1951) by coating them with 0.5% celloidin before infiltration and embedding in Paraffin wax. The embedded specimens were sectioned at 5 µm in thickness. The sections were stained in Erlische's haematoxylin and counter-stained with eosin. Microscopic examination of the sections took place and good sections were then photographed.

RESULTS AND DISCUSSION

The histopathological patterns revealed variable effects on *G. mellonella* larvae among fungus, nematodes, fungus/heterorhabditid and fungus-steinernematid treatments. In all cases of nematodes involvement in the infection, muscles and fat tissues were greatly affected in comparison with those of control tissues (Fig. 1). Muscles suffered destruction in the fibrillae with some fragmentation (Fig. 2), complete destruction and dissolution (Fig. 3) and fat tissues showed high vacuolation (Fig. 4, Fig. 5). Similar results have been documented by Soliman (2002) concerning the effects of *H. bacteriophora* and *S. carpocapsae* on *Ceratitis capitata* larvae. The depletion of fat body might result from catabolism stimulation of the fat-body protein which will be utilized by the nematode for its growth, as reported for mermethids by Tanada and Kaya (1993).

Cases of cuticular and respiratory system fungal invasion (Fig. 6, 6*) were observed in the case of *B. bassiana* alone, where strong melanization appeared together with an aggregation of hemocytes, three days postinfection. This agreed with findings reported by Ferron (1985) that hyphae of *B. bassiana* triggered a flux of hemocytes at the area of penetration. Vey and Götz (1986) explained that the aggregation of the blood cells starts after the rapid melanization that occurred at the inner surface of the respiratory organs. No encapsulation around the fungal cells has been observed in the present study. This is in consistence with the observations of Vey and Fargues (1977) in the infection of *Leptinotarsa decemlineta* larvae with *B. bassiana*.

Encapsulation around the nematodes with or without involvement of hemocytes (Fig. 7) was seen in the case of the treatment with *Steinernema* alone but not with heterorhabditids. No cases of melanization in the infected larvae were observed with both nematodes. These findings agreed with those of Stoffolano and Yin (1986). They reported that penetration of the cuticle or epidermal cells by nematodes may elicit a tissue response by the host. Hemocytic encapsulation is involved in the defense mechanism against nematodes which is sometimes accompanied by melanization with or without participation of the hemocytes (Poinar, 1974; Götz *et al.*, 1977).

Variations in the development of the fungus-nematodes infection between *H. bacteriophora* and *Steinernema* sp. treated groups were recorded. It was difficult to detect the fungal cells or hyphal bodies in the larvae treated with *B. bassiana* and *H. bacteriophora,* whereas with *Steinernema* sp., fungal propagules were found in almost all sections (Fig. 8). This may explain, in a previous study (Zayed, *et al.*, 2003), why no fungal growth was detected during the first 3 days on *G. mellonella* cadavers simultaneously pre-treated with *B. bassiana* and *H. bacteriophora* when using the same concentration applied in the present study. However, the same treatment with *Steinernema* sp. resulted in fungal growth in almost all the cadavers. Fungus-*Steinernama* combined treatment did not initiate the formation of hemocytic or humoral encapsulation of nematode as in the case of nematode alone. The previous finding might be due to the exhaustion of hemocytic response of *G. mellonella* in the presence of the fungus. More conceivable, the mycotoxins produced by *B. Bassiana* might inhibit encapsulation by the immunodepressive effect. Similarly, the mycotoxin destruxin E1 increased the susceptibility of *G. mellonella* to *Aspergillus niger* by pursuing the same manner (Vey *et al.*, 1985). Destructed and heavily melanized steinernematids have been found in the sections particularly 3 days post dual-infection (Fig. 9). This might support our previous study (Zayed *et al.*, 2003) in which inhibition of steinernematid recycling in *G. mellonella* cadaver was noticed. Some cases of nodule formation and melanization around hyphal cells of *B. bassiana* were observed (Fig. 10). In contrast to fungus-steinernamatid infection, fungus-heterorhabditid infection showed no cases of encapsulation. However, coagulation of humoral strands beside groups of plasmatocytes was observed in the first day post-infection distant from the nematode (Fig. 11) and aggregation of hemocytes was triggered towards the nematode on the second day of infection (Fig. 11*). The fungus-heterorhabditid infection caused great damage in the larval body on the third day post infection (Fig. 12, 12*) that rendered manipulation and observations difficult.

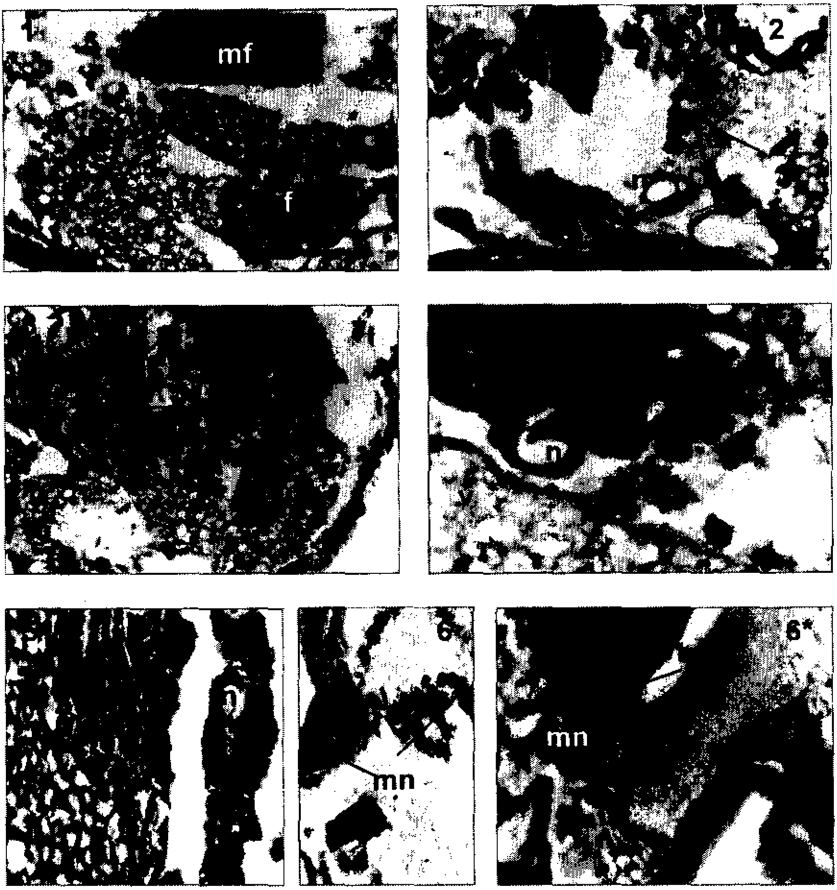

FIG 1. Cross section of untreated *G. mellonella* last instar larva shows intact muscle fibres (mf)and fat tissues(f). x 400.

FIG. 2. Cross section of fungus-steinernematid 3-days infected larva shows fragmentation of muscle fibres (mf) and depleted fat cells. Note melanized nematodes (n) and fungal cells (fc). x400.

FIG. 3. Cross section of fungus-heterorhabditid 3-days infected larva shows complete destructed muscles (mf) and depleted fat cells(f). x150.

FIG. 4. Cross section of fungus-heterorhabditid 2-days infected larva shows high vacuolation (v) in the fat cells (fc). x1000.

FIG. 5. Cross section of fungus-heterorhabditid 3-days infected larva shows obliteration of fat cells (f). x400.

FIG. 6. Longitudinal section of fungus 3-days infected larva shows cuticular invasion resulting in some destruction of the hypodermal cells. Note the melanized areas (mn) adhering to the fungal cells (fc). x400.

FIG. 6*. Longitudinal section of fungus 3-days infected larva shows respiratory invasion (trachea:t). Note the flux of granulocytes (gr) engaged in nodule formation and the heavy melanization (mn) adjacent to fungal cells. x 400

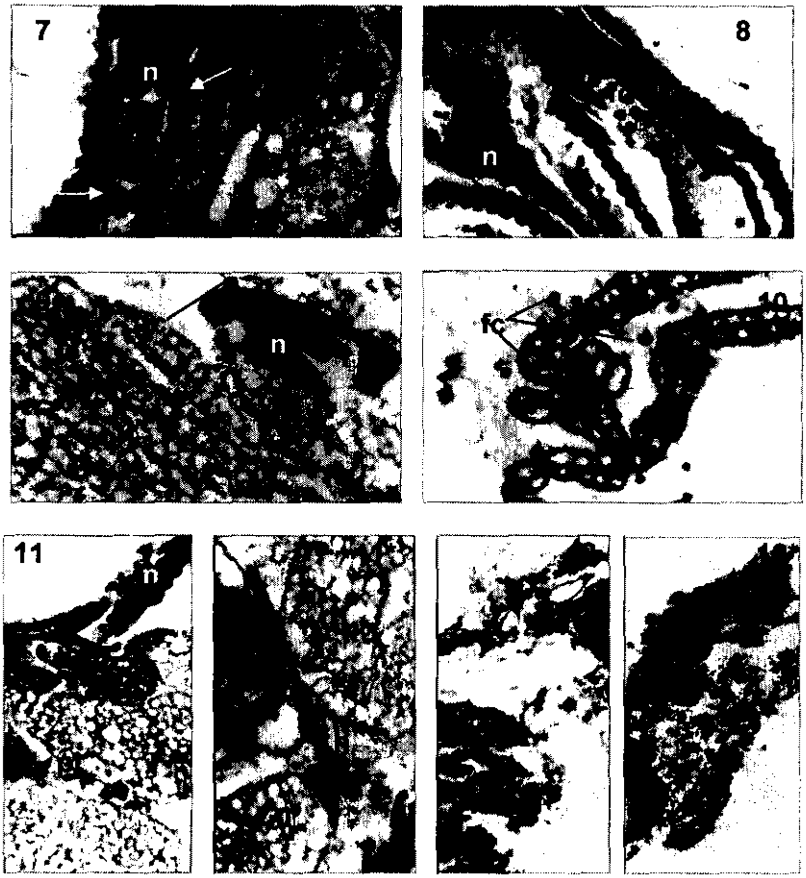

FIG. 7. Longitudinal section of steinernematid 2-days infected larva shows encapsulation of nematode (n). x 400.

FIG. 8. Longitudinal section of fungus-steinernematid 3-days infected larva shows dual invasion of fungus (fc) and penetrating nematode (n). x400.

FIG. 9. Destructed and melanized *Steinernema* (n) in presence of fungal cell in 3-days infected larva. x1000.

FIG. 10. Nodule formation on fungal cells (fc) in 3-days infected fungus-steinernematid larva. x400.

FIG. 11. Cross section of fungus-heterorhabditid 2-days infected larva shows clumps of plasmatocytes (pl) near to humeral coagulation (hm) towards nematode (n). x400

FIG11*. Longitudinal section of fungus-heterorhabditid one-day infected larva shows hemocytes (h) aggregation around penetrating nematode (n). x400.

FIG12. Longitudinal section of fungus-heterorhabditid 3-days infected larva shows complete destruction of body tissues x 100.

FIG. 12*. detached cuticular layer as well as damaged epidermal cells. x400

This variation of insect defense mechanisms against fungus, nematodes, and both pathogens together in the larval body might be due to the diverse interactions resulting from multiple infections. The different organisms can develop simultaneously or successively in the insect host during this coexistence, antagonism or synergism might occur (Vago, 1963).

SUMMARY

Interaction between the entomopathogenic fungus *Beauveria bassiana* and the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema* sp. revealed variable histopathological effects on *G. mellonella* last instar larva. The presence of the fungus impeded both cellular and humoral encapsulations of *Steinernema* sp. by the insect defence mechanism. However, *B. bassiana* inhibited the development of *Steinernema* sp. by initiating the melanization process and help in the destruction of nematodes in the larval body which was not the case by *H. bacteriophora*. This study elucidated why *Steinernema* sp. failed to reproduce in the larval cadaver in the presence of *B. bassiana* and affirmed that *H. bacteriophora* might tolerate fungal presence more.

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