MORPHOLOGICAL ASPECTS OF CORN EARWORM (*HELIOTHIS ZEA*) INFECTIONS BY THE FUNGUS *BEAUVERIA BASSIANA*

By

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MORPHOLOGICAL ASPECTS OF CORN EARWORM (HELIOTHIS ZEA) INFECTIONS BY THE FUNGUS BEAUVERIA BASSINANA

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PREFACE

This study was concerned with the mode of entry of Beauveria bassiana into the corn earworm (Heliothis zea) and the consequential histological ramifications. An understanding of these phenomena may uncover a means of enhancing the efficiency of the pathogen. The primary objective was to determine the location and nature of the initial penetration by B. bassiana into and proliferation within first instar corn earworm larvae using both light and scanning electron microscopy.

I wish to express my sincere gratitude and appreciation to Dr. E. A. Grula for his patient guidance, support, and encouragement throughout this endeavor. Appreciation is also extended to Dr. Robert Burton for his counsel and assistance in understanding the morphology and maintenance of the corn earworm larva.

I wish to convey my deep gratitude and appreciation to David Cockrell and my parents, Hart and Barbara Pekrul, for their continual friendship, understanding and encouragement.

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CHAPTER I

INTRODUCTION

The fungus, Beauveria bassiana, has been recognized as an entomopathogen since 1835 when Agostino Bassi proved it to be the organism responsible for white muscardine of the silkworn (Pramer, 1965.) Since then it has been found to be a widely distributed, nonspecific entomopathogen (Barson, 1977). As such, it is a potential biological insecticide.

Conidia of B. bassiana are globose to oval and vary greatly in size with an average diameter of approximately 3 μm (MacLeod, 1954). These conidia are among the smallest in the fungi (Hawker and Madelin, 1976) and have been reported to be unornamented (Mangenot and Reisinger, 1976; Reisinger and Olah, 1974). Primary spores are produced on conidiophores which may be simple or branched; these structures vary greatly in size, shape, and number of conidia produced (MacLeod, 1954). Secondary spores and blastospores are produced directly on the mycelium and are more cylindrical (7–12 μm by 3–3.5 μm) than conidia (MacLeod, 1954).

On germination, conidia swell and produce 1–3 germ tubes. These may protrude from any place on the spore (amphigeous). Septate hyphae will increase in width with age and, hence, vary in diameter from 1.5–3.5 μm (MacLeod, 1954).

Infection by B. bassiana has generally been regarded as a result of direct penetration of hyphae through the integument (Lefebvre, 1934;

Vey and Fargues, 1977; Ferron, 1978; Pramer, 1965; and Steinhaus, 1949) which is facilitated by both mechanical and enzymatic activity. However, penetration through the alimentary tract (Broome et al., 1976; and Gabriel, 1967) and the respiratory system (Clark et al., 1968; Hedlund and Pass, 1968; and Steinhaus, 1949) have also been reported.

It was interesting to note the integument is the most commonly noted route of infection as it appears to project the most formidable barrier. The integument is composed of three layers, the epicuticle, exocuticle, and endocuticle, as shown in Figure 1 (Chapman, 1969).

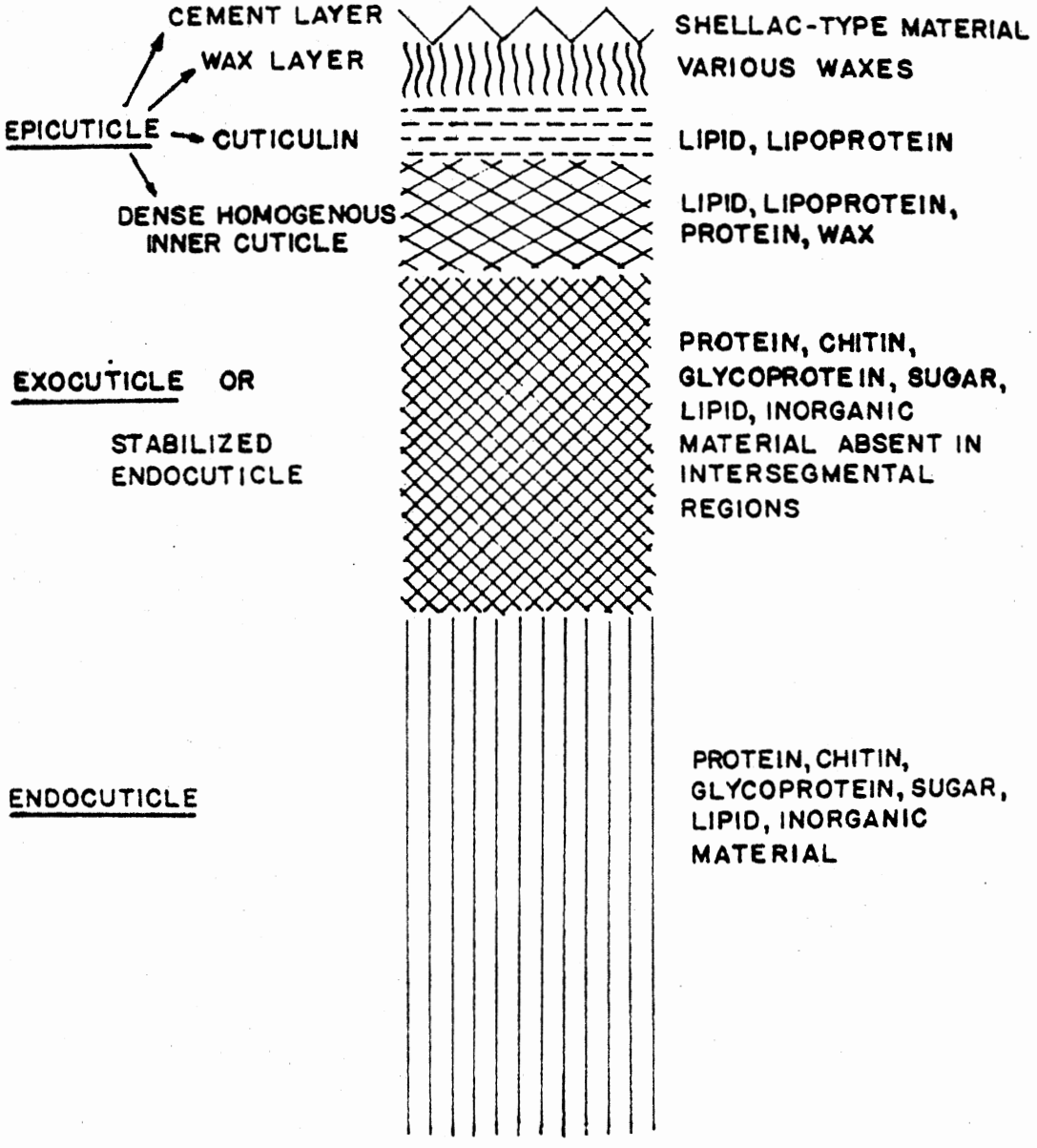
The epicuticle itself can have four layers and covers the entire insect. The outermost layers are the cement layer, containing a shellac-type material, and a wax layer (Hackman, 1974). The cuticulin, the third layer, consists of stabilized lipid which can bind to protein to stiffen the cuticle (Wigglesworth, 1972). The dense, homogenous inner cuticle layer appears to contain lipid, lipoprotein, and wax (Hackman, 1974).

The bulk of the cuticle consists of the exocuticle and endocuticle which are primarily protein and chitin. Almost half of the dry weight of the cuticle is protein alone; its amino acid composition is the same throughout the cuticle. Chitin, unbranched chains of β-(1,4) N acetyl-D-glucosamine, is often found complexed with protein. These glycoproteins consist of rods of chitin surrounded by protein, which are bound by both covalent and weaker bonds (Hackman, 1974).

Sclerotization (hardening of the cuticle) in the exocuticle occurs when proteins are crosslinked by reacting with quinones (Hackman, 1974). Consequently, both protein and chitin become more highly oriented. The intersegmental regions lack these sclerotinous proteins.

Figure 1. Diagram of the Insect Integument Composition

INSECT INTEGUMENT



Penetration of the integument by entomopathogenic fungi has been reportedly accompanied by a dissolution of the cuticle (Lefebvre, 1934; McCauley et al., 1968) indicating enzymatic activity. It has also been reported that mechanical forces are involved in penetration (Lefebvre, 1934; Yendol and Paschke, 1965). Penetration usually occurs at the intersegmental regions (Lefebvre, 1934; McCauley et al., 1968) where sclerotization has not occurred. Once the infection is in progress, the reported order of tissues infiltrated by the fungus varies. Dermal cells may be destroyed near the point of penetration (Steinhaus, 1949), but the fat body is usually attacked first (Lefebvre, 1934; Yendol and Paschke, 1965; McCauley et al., 1968; Yen, 1962). Disintegration of muscle, nerves, and glandular structures may follow (Lefebvre, 1934; Yen, 1962; Yendol and Paschke, 1965); however, upon larval death, these structures may still be intact along with the alimentary tract and tracheae (Yen, 1962; Yendol and Paschke, 1965). Since death can occur with little tissue damage, it has been speculated that toxins or alterations of the hemolymph are involved (McCauley et al., 1968).

Entry of the fungus through the respiratory system is another possibility. This type of invasion is reported to occur by penetration through tracheae adjacent to spiracles (Clark et al., 1968; Hedlund and Pass, 1968). However, nonpenetrating germ tubes have been observed within tracheae (McCauley et al., 1968). Death may be due to toxin production or mechanical blockage of tracheae (Clark et al., 1968).

The enzymes necessary for penetration of the tracheae would probably be proteases and/or chitinases. The tracheae are formed by invaginations of the ectoderm and hence, are composed of cuticle similar to that of the larval surface. Its procuticle, though, is much thinner

and it contains no cement or wax (Hackman, 1974). Consequently, penetration of the tracheae and integument could involve similar or identical enzymes.

The last possible route of infection is the alimentary tract. In this case, an invading germ tube would need to penetrate the enteric epithelium which is lined by a cuticular layer of chitin and protein (Snodgrass, 1935). Again, a chitinase and/or protease could be involved. One important requirement for this type of infection is that conidia must survive the high pH of the gut, which for the corn earworm is pH 8.6 to pH 9.27 (Burton, unpublished). Microfeeding of conidia to larvae has yielded some data on this problem. Penetration of the gut wall has been reported (Broome et al., 1976); however, germination inhibition is evident (Gabriel, 1967). Germination before ingestion may be necessary for survival (Yendol and Paschke, 1965).

Most of the studies of entomopathogens have been based on light microscopy; a few notable ones have been made using electron microscopy. A study of infection by Metarrhizium anisopliae with transmission electron microscopy (TEM) revealed that penetration occurs through the integument by appressoria (Zachuruk, 1970a,b). Appressorial cells were found to contain numerous mitochondria, dictyosomes, ribosomes, and endoplasmic reticulum, suggesting a high level of metabolic activity. Zacharuk concluded the penetration was primarily enzymatic in nature, but was initiated by mechanical activity. A later study of M. anisopliae using scanning electron microscopy (SEM) revealed that germ tubes grow extensively over the cuticle before forming appressoria (Schabel, 1978). Appressorial-like structures were also found using TEM in an infection of Leptinitarsa decemlineta by B. bassiana (Vey and Fargues,

1977). All three of these electron microscopic studies emphasize the formation of specialized structures before enzyme production.

The target organism of the present study, the corn earworm (Heliothis zea), is the most important pest of corn and the second most destructive insect in the United States (Swan and Papp, 1972). Also known as the cotton bollworm and tomato fruitworm, this insect can be found world-wide feeding on a variety of vegetables and flowers. Adults emerge in the spring from overwintering pupae and lay eggs throughout the summer. The life cycle is only about 35 days; therefore, 3 or more generations may occur annually.

In the present study, infections of Heliothis zea by mutants of B. bassiana will be observed using light microscopy and SEM. Scanning electron microscopy is essential in understanding the nature of fungal growth on the larval surface and the route of infection. The later internal stages of infection are best revealed by light microscopy. It is hoped that understanding the infection process of B. bassiana will lead to methods for enhancing the entomopathogenicity of this fungus.

CHAPTER II

MATERIALS AND METHODS

Test Organism

Mutants of Beauveria bassiana (Vuill.) were obtained in this laboratory by irradiating (UV) conidial suspensions and selecting for differential proteolytic capabilities on Sabourauds dextrose agar (SDA, Difco) containing litmus milk (casein protein). Some mutants were collected from larval passages (i.e., from a mummified larva). Pathogenicity of these mutants toward the corn earworm was determined by probit analysis in this laboratory. A pathogenicity ranking of the mutants according to LD_{50} and LC_{50} values is given in Appendix A.

Larval Colony and Maintenance

Corn earworm larvae (Heliothis zea) used in this study were obtained from the USDA Entomological Research Laboratory, Stillwater, Oklahoma. Larvae were grown at 25°C on Burton's CSM diet (Burton, 1970) and a modification of this diet in which D-chloramphenicol (0.5 mg/3.8 L) replaced the antifungal agents (formaldehyde, sorbic acid, and p-hydroxymethybenzoic acid) normally employed.

Media

Cultures of Beauveria bassiana were grown on SDA slants at 25°C. Transfers were made only from sporulated cultures. Frequent transfers

(every 3 weeks) were necessary to maintain cultures. Storage was at 4°C or at room temperature.

Submerged cultures were grown for 7 days in Sabourauds dextrose broth (SDB) aerated on a rotary shaker.

Preparation of Conidia for Infection

Conidia from 7 day old cultures of B. bassiana grown on SDA plates were harvested by two methods. The first involved washing the conidia from the cultures with 0.1% Triton X-100. This suspension was then centrifuged and washed 3 times with distilled water. Small volumes (1-2 ml) of the resulting suspension were then applied to sterile filter paper in a petri dish.

In the second method, sterile filter paper was placed on fungal cultures and either dampened with 1-2 ml distilled water or left dry. After ten minutes, the paper was inverted and placed in a sterile petri dish.

For both collection methods, no attempt was made to apply a constant amount of conidia to each larva.

Infection Process

First instar larvae were allowed to crawl for 10-20 minutes on filter paper covered with conidia. During this time, an effort was made to keep the larvae on the filter paper, but this was not always possible. Larvae were later placed on regular or modified CSM diet. In order to conserve diet, as many as 4 larvae were placed in a cup. Larvae not treated with conidia were grown on modified and regular CSM diet as controls.

Light Microscopy

Preparation of Larvae

Infected larvae and controls were killed and fixed at various time intervals following infection with alcoholic Bouin fixative at 60°C for 2 hours (Grimstone and Skae, 1972). Following a 24 hour wash in 70% ethanol, larvae were dehydrated and embedded in paraffin using a modification (see Appendix B) of Zirkle's tertiary butanol series (Zirkle, 1934). Sections of 4-10 μm thickness were obtained using a Spencer rotary microtome and fixed to glass slides with Haupt's solution (Berlyn and Miksche, 1976). Modifications of Hamm's stain and Mayer's hemalum stain were used (Hamm, 1966; Grimstone and Skae, 1976). The staining formulae and sequences are provided in Appendices C and D. Stained sections were then examined and photographed using a Nikon phase contrast microscope and Panatomic-X film.

Preparation of Fungi

Mycelia and conidia were observed in smears stained with lactophenol cotton blue and in paraffin-embedded samples prepared as described above.

Scanning Electron Microscopy

Preparation of Larvae

Infected and control larvae were killed and fixed for 2 or more hours at room temperature in 4% glutaraldehyde buffered with 0.2 M cacodylate buffer at pH 7.3. After washing with buffer for 1 hour,

larvae were postfixed for 1-2 hours with 1% osmium tetroxide (Hayat, 1970). Dehydration was in an ethanol series (30-100%) with each step lasting 15-20 minutes. Care was taken not to aggitate the larvae so that conidia on the surface would not be disturbed. The samples were then critical point dried using liquid CO_2 and mounted on aluminum stubs with silver conducting paint or double adhesive tape. The larvae were plated with gold-palladium for 2 minutes using a Hummer evaporator; this resulted in a deposition of approximately 400Å (Hayat and Zirkin, 1970). Examination was accomplished using a JOEL 35 JSM scanning electron microscope at an accelerating voltage of 25KV. Photographs were made using Polaroid film.

Preparation of Fungi

Suspensions of conidia were prepared from cultures grown on SDA by washing with water or 0.1% Triton X-100. Spores from broth cultures were collected by filtration through cheesecloth. Spores were placed on membrane filters (pore sixe 0.4 μm, Bio-Rad Laboratories) by filtration or immersion. The samples were then fixed, dehydrated, critical point dried, plated and examined as described previously.

CHAPTER III

RESULTS

Surface Morphology of *Beauveria bassiana*

Conidia from seven day cultures vary from globose to oval (average diameter of 2.13 μm) and from unornamented to slightly ornamented. Mutants differ somewhat in these qualities. For example, mutant 8 is usually more ornamented and oval than other mutants such as R_1 and 9 (Figures 2-4). However, conidia produced by each individual mutant also vary in shape and ornamentation. Consequently, mutants cannot be identified by surface morphology of their conidia.

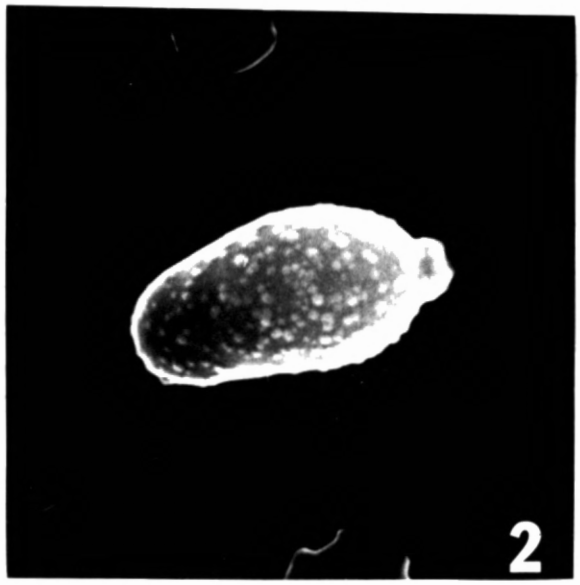
Conidia surface texture can be altered slightly by washing with Triton X-100 (Figures 4 and 5). Ornamentation is occasionally increased, but still varies among individual mutants.

When grown in broth cultures, *B. bassiana* produces blastospores which differ substantially from conidia. Blastospores are elongated (as long as 26 μm) and bleakly ornamented (Figure 6).

Upon germination, conidia swell and produce 1 or more germ tubes. These germ tubes can protrude from any point of the spore with respect to the birth scar. No special cellular structures or texture differences were observed at the hyphal tip.

Figure 2. Conidium From 7 day Culture of Mutant 8 Grown on SDA. The conidial suspension was prepared by washing the culture with water. Structures in the background are the pores of the membrane filter. 12,000x.

Figure 3. Conidia from 7 day Culture of Strain R1 Grown on SDA and collected with water. 15,000x.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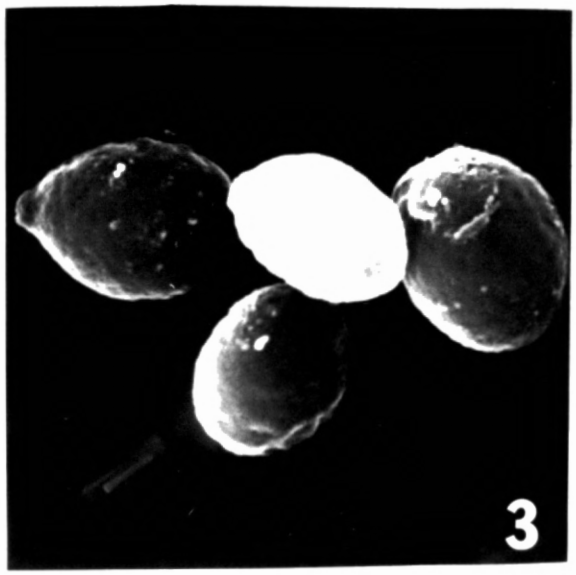
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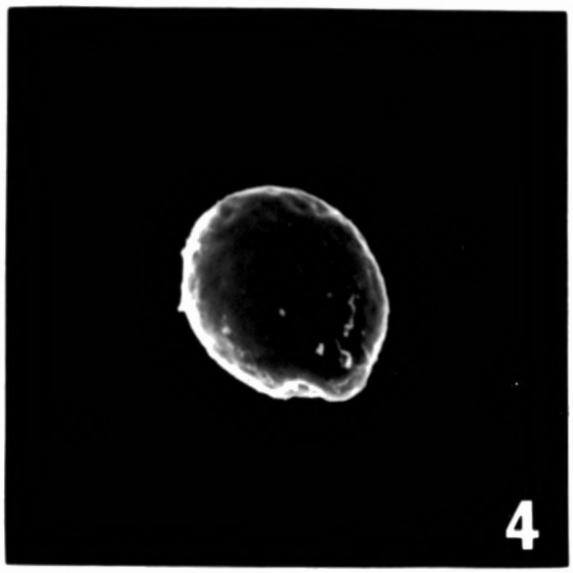
Figure 4. Conidium From 7 day Culture of Mutant 9 Grown on SDA and collected with water. 12,000x.

Figure 5. Conidia From 7 day Culture of Mutant 9 Grown on SDA and collected with Triton X-100. Note the increased ornamentation compared to water-washed mutant 9 conidium. 12,000x.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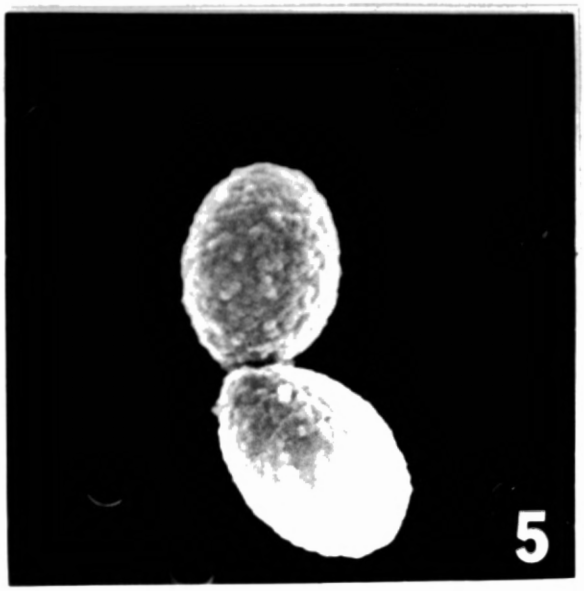
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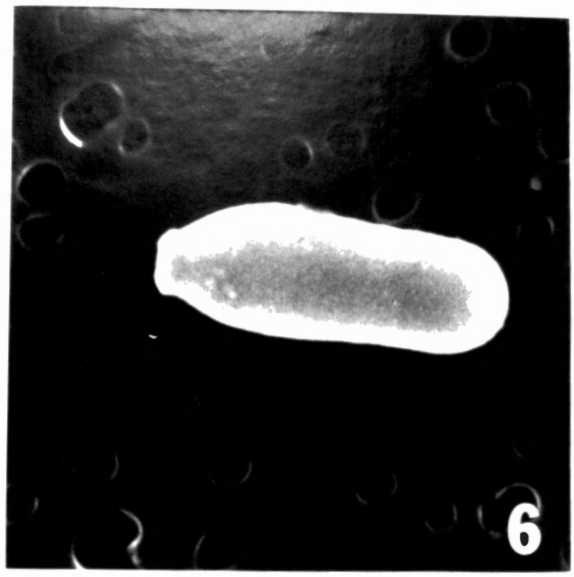
Figure 6. Blasostopore From 7 day Culture of Mutant 9 Grown in SDB. Note the elongation and lack of ornamentation. 12,000x.

Figure 7. Germinating Condium of Mutant E1. This sample was fixed 15 hours after the broth was inoculated with conidia from a 7 day culture. Note the outer wall fragment. 12,000x.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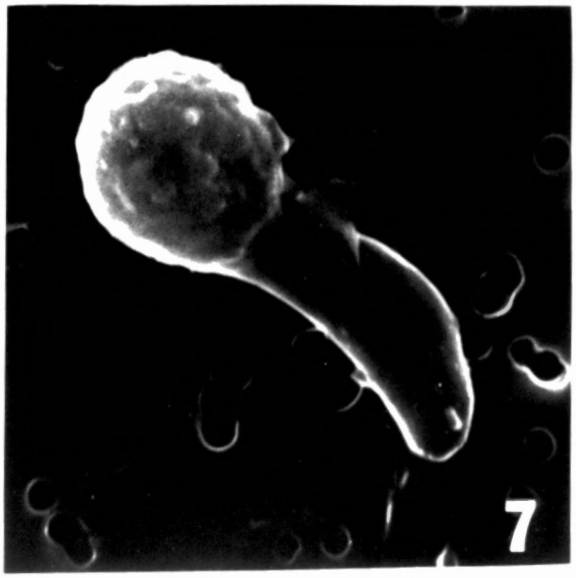
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Figure 8. First Instar Corn Earworm Larva. Spiracles (black arrows) and setae (white arrows) are quite noticeable. 100x.

Figure 9. Surface Features of First Instar Corn Earworm Larva. Included are nodules (n), a seta (s), and a spiracle (sp). Note the angular geometry of the artifactual wrinkling of the cuticle (a). 1200x.


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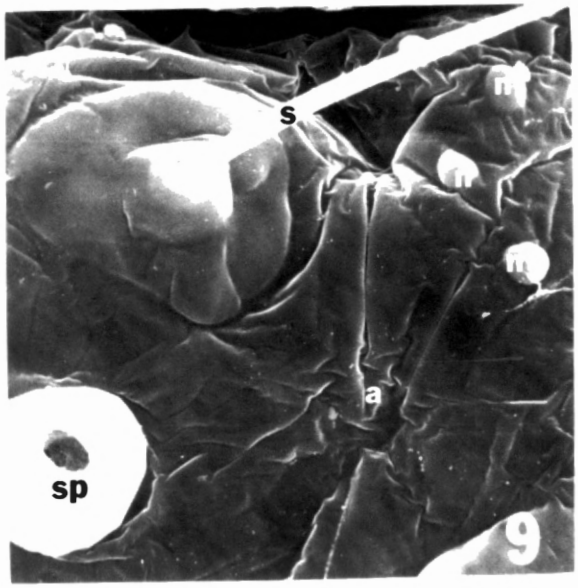
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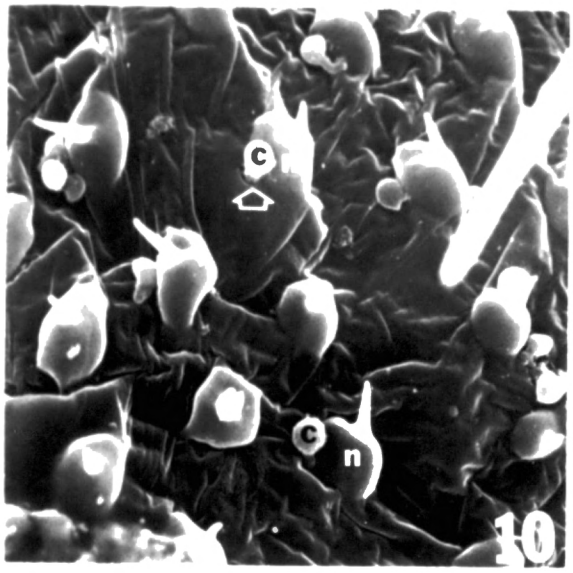
Figure 10. Distribution of Conidia (c) Near Nodules (n). Enzymatic degradation of the integument is shown by the hole at the point of penetration by a germ tube (arrow). The conidia are of Mutant E1. (This sample was fixed 18 hours after infection). 2000x.

Figure 11. Penetration of the Integument (arrows) at the Base of Nodules (n). Mutant E1. (24 hours post-infection) 2000x.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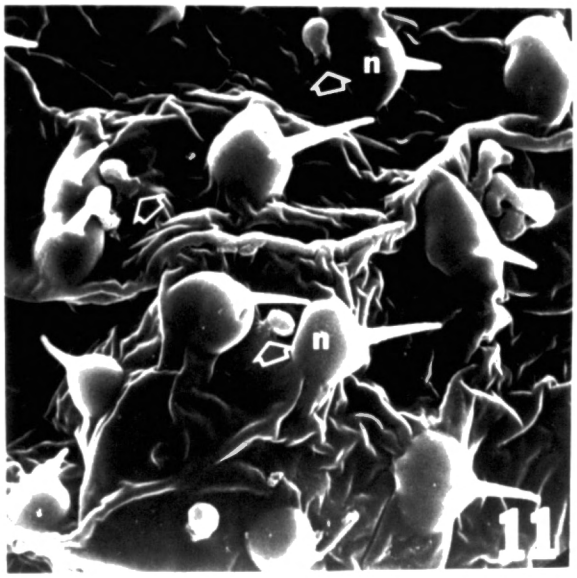
n
n
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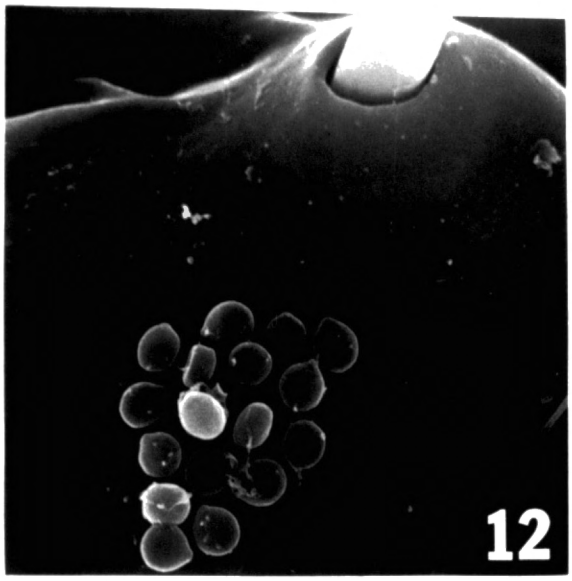
Figure 12. Cluster of Conidia at the Base of a Seta. (Mutant E1) 3600x.

Figure 13. Heavily Infected Larva with Many Germinating Conidia. Note the hole in the integument (arrow). Most germ tubes are oriented toward penetration. Mutant E1. (30 hours) 2000x.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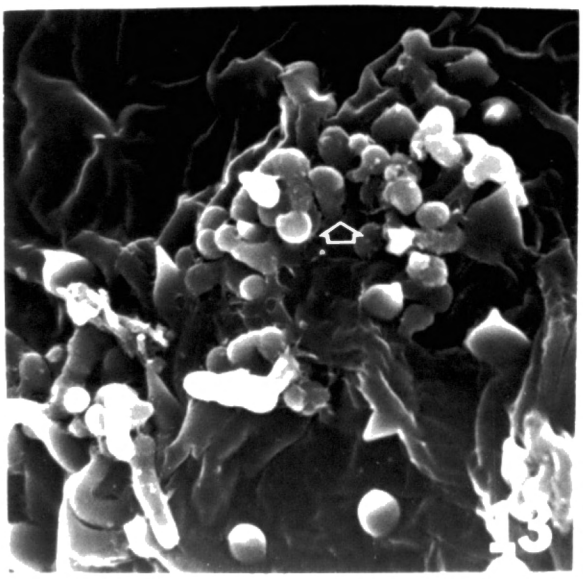
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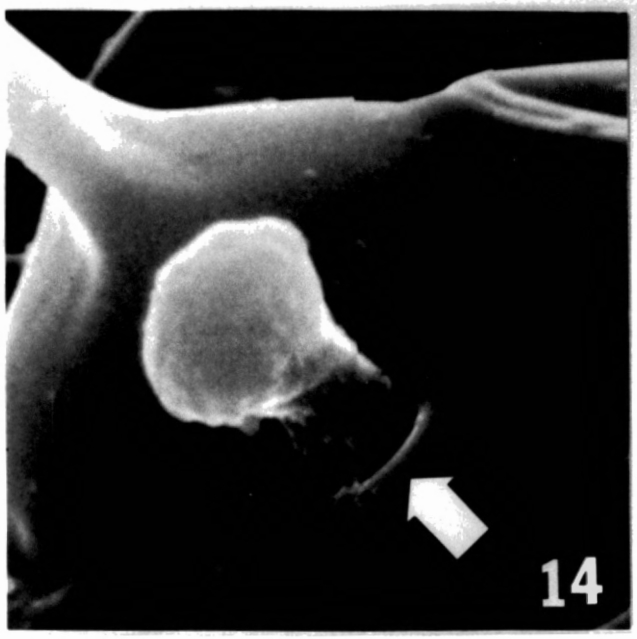
Figure 14. Enzymatic Degradation and Production of a Hole in the Integument by an Invading Germ Tube (arrow). Mutant E1. (18 hours) 7800x.

Figure 15. Strong Orientation of Germ Tubes Toward the Integument. Penetration does not always occur at the base of nodules and some penetrating hyphae exhibit a slight increase in width (w). Formation of a hole is occurring at the base of one penetrating hypha (arrow). Mutant E1. (24 hours) 3600x.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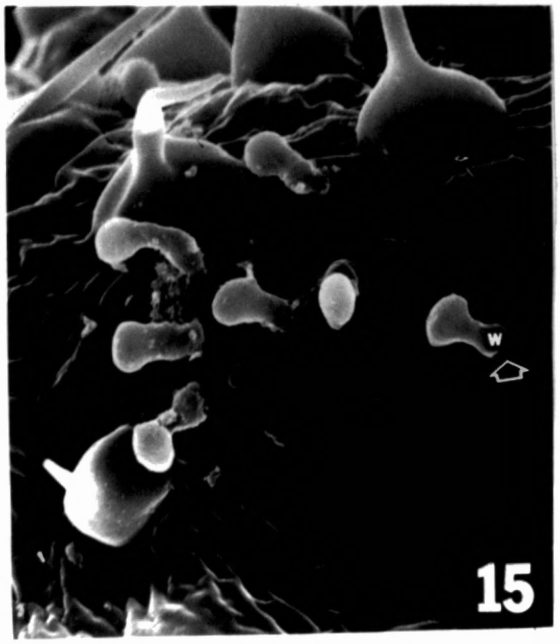
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and were able to penetrate that structure soon after germination. Penetration was observed as early as 18 hours after infection (Figures 10, 11, and 14). Penetrating hyphae had an average visible length of 1.4 μm. Due to the location of the conidia, penetration usually occurred at the base of nodules (Figures 10, 11, and 14), but could be observed elsewhere (Figure 15).

Not all germinating conidia of mutant E1 produced penetrating germ tubes. Clusters of germinating conidia commonly yielded some errant hyphal growth (Figure 13). Dispersed conidia exhibited a greater tendency to produce penetrating germ tubes, but even some isolated conidia failed to penetrate soon after germination (Figures 10 and 11).

Penetration through the cuticle occurs without the formation of appressorial structures. There is an occasional slight thickening of the germ tube just prior to penetration (Figure 15), but this is not enough to signify appressorial formation. A hole in the cuticle was often visible around the germ tube at the point of penetration (Figures 13, 14, and 15).

Germinating conidia were sometimes present on the head of heavily infected larvae, but penetration was not evident (Figure 16). Instead, hyphae grew extensively with no apparent orientation.

In addition to direct penetration through the integument, the fungus may enter through the respiratory system. B. bassiana conidia are small enough to be trapped within a spiracle atrium and are able to germinate there (Figure 17). Germ tubes are also able to enter the atrium and to penetrate the spiracle proper (Figure 18).

Mummified larvae reveal fungal growth protruding first at the intersegmental regions but eventually appearing over the entire insect

Figure 16. Hyphae From Germinated Conidia Growing Errantly and Unable to Penetrate the Head. Mutant E1. (20 hours) 360x.

Figure 17.. Germinating B. bassiana Conidia Within a Spiracle. Mutant E1. (20 hours) 6000x.

Figure 18. Germ Tubes Entering the Spiracle Opening (g). Note the enzymatic degration at the edge of the opening near the germ tube (black arrow). A germ tube is also penetrating through the side of the spiracle (white arrow). Mutant E1. (30 hours) 1600x.


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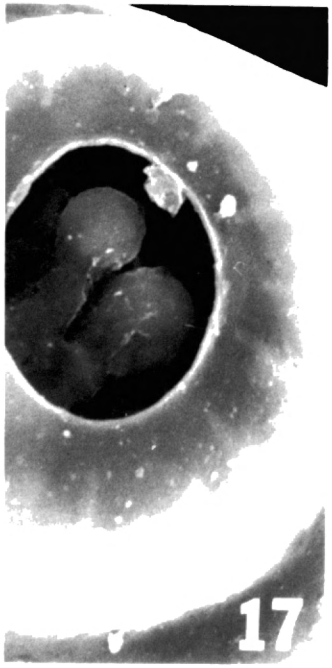
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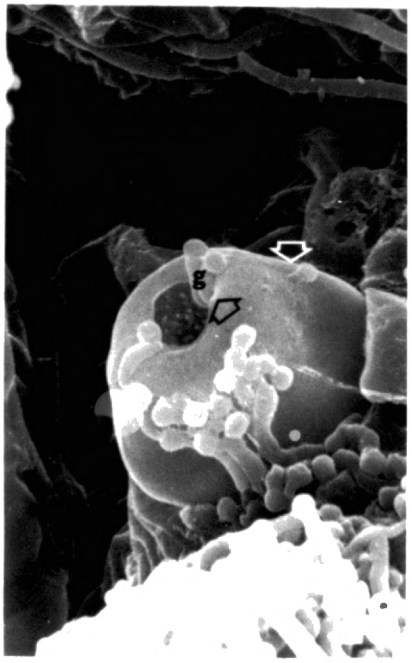
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(Figure 19). The integument did not appear to be degraded at the point of hyphal exit as it did upon penetration (Figure 20). Rather, the integument was almost indistinguishable from the protruding hyphae. No hyphal growth appeared from the spiracles.

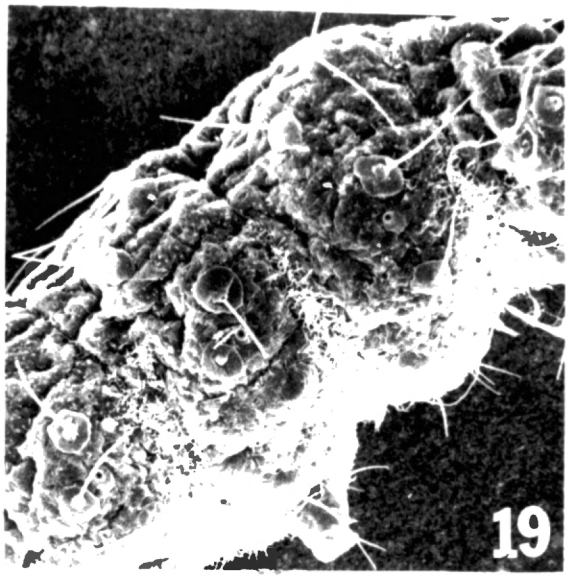
Light Microscopy

With the staining methods used, various organs and structures could be distinguished in thick cross-sections of first instar larvae (Figure 21). The cuticle, including nodules, appeared somewhat transparent with varying thicknesses (Figure 22). Sections through setae and spiracles were rarely observed. The epithelium is a single layer of dense cells (Figure 22) directly beneath the cuticle. Silk glands stain very darkly (Figure 23), whereas the muscle and mid-gut are much lighter (Figures 21 and 22). The fat body is a loosely-bound structure with various staining qualities (Figures 21 and 22); some areas of the fat body are quite dense while adjacent regions may appear extremely lacy. The staining quality of tracheae is similar to that of the cuticle (Figure 23). The ribbed texture of tracheae is often noticeable. Malpighian tubules may be mistaken for tracheae except for their irregular shape and thickness (Figures 21 and 22). The hemocoel of uninfected larvae is more easily noticed due to numerous empty areas (Figure 21). Basement membranes, peritrophic membranes and hemocytes were difficult to distinguish with consistency.

Penetration of the cuticle was rarely observed in thick sections, even in larvae infected with a high concentration of conidia. When observed (Figure 22), the length of germ tubes exterior to the cuticle was comparable to those observed by SEM.

Figure 19. Mummified Larva. Note the fungal growth in the intersegmental regions and the lack of growth from the spiracles. Mutant E1. (6 days) 660x.

Figure 20. Hyphae Protruding From a Mummified Larva. Note the lack of an enzymatic hole (arrow). Mutant E1. (11 days) 1100x.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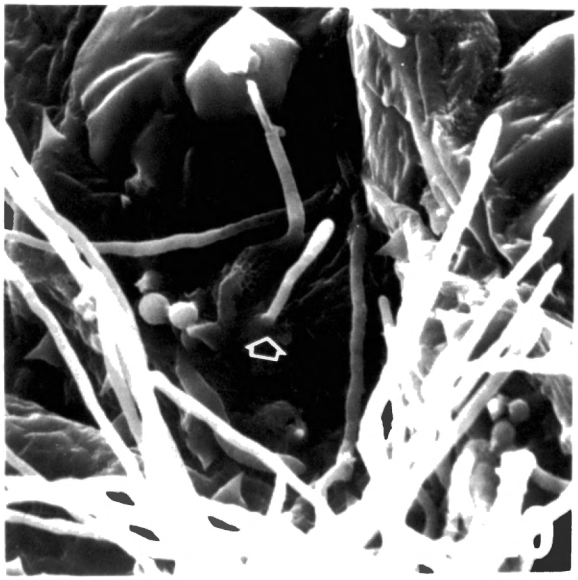
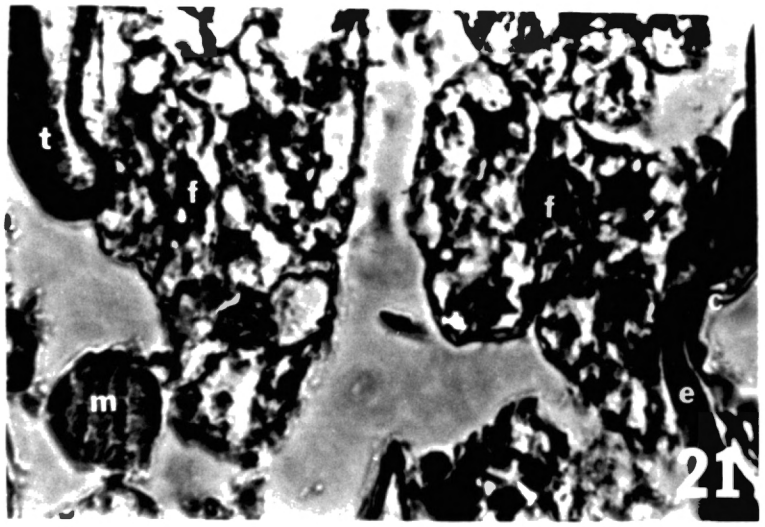


Figure 21. Thick Section of an Uninfected First Instar Corn Earworm Larva. Note the appearance of cuticle (c), muscle (m), epithelium (e), fat body (f), gut tissue (g), and Malpighian tubules (t). 1400x.

Figure 22. Thick Section of a Germ Tube Penetrating the Cuticle (arrow). Fat body (f) and epithelial cells (e) are evident. Mutant E1. (22 hours) 1400x.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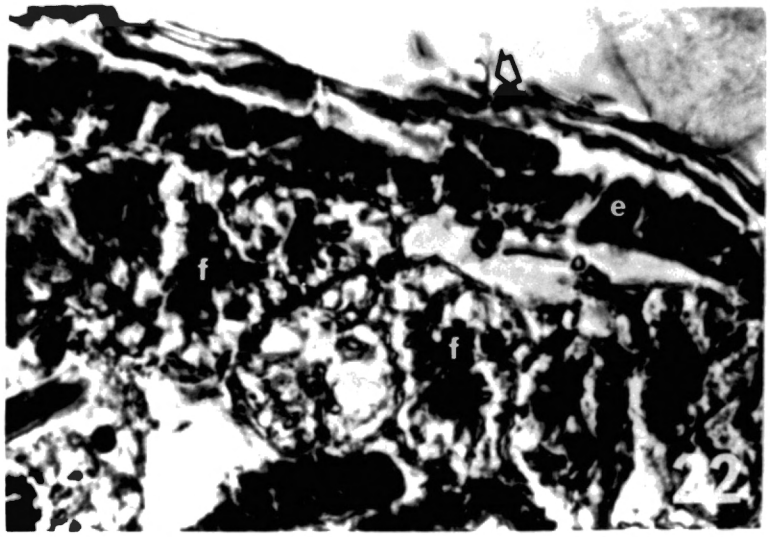

e
f
f
22

Extensive fungal growth is evident in the hemocoel within 48 hours after exposure to conidia (Figure 23). This growth takes place with no apparent tissue damage. The fat body was the first tissue to be infiltrated; this occurs about 60-72 hours after infection. On the fourth to sixth day, gut tissue and Malpighian tubules show signs of degradation; mummification has usually begun by this time. Tissues which appear unaffected, even in extensively mummified larvae, are the silk glands, muscle, and tracheae (Figure 24).

Hemocytes could be observed (with difficulty) in all stages of the infection. However, due to extensive fungal growth in the hemocoel, relative numbers of these cells could not be determined.

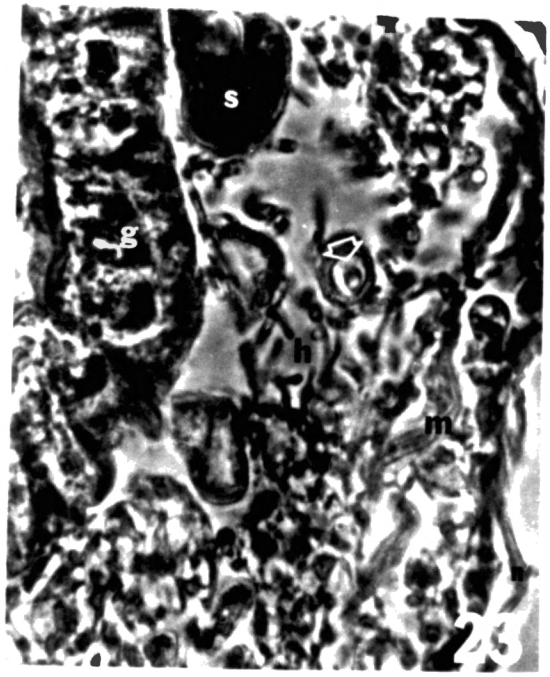
Mummification could occur without any apparent tissue damage (Figure 24). The hyphae could be protruding through the cuticle while the gut, muscle, tracheae, silk glands, and some of the fat body were still intact. At this stage, the hemocoel was completely filled with mycelial growth. It seems probable that death could occur without excessive tissue damage.

Less Pathogenic Mutants

On the larval surface, the behavior of mutant E1 is different from that exhibited by less pathogenic mutants. Conidia of some mutants, such as #3, fail to adhere well to larvae even if a high concentration of conidia are used to infect. Also, nongerminating clusters of conidia can be observed (Figures 25 and 26). Conidia that germinate do yield hyphae that are highly oriented toward penetration (Figure 27). Instead, the majority of conidia produce hyphae which grow extensively over the larval surface in an errant manner (Figures 28 through 30).

Figure 23. Extensive Fungal Growth in the Hemocoel (h) with no visible damage to the gut tissue (g), silk glands (s), and muscle (m). A hypha is visible within a tracheae (arrow). A nodule (n) is also present. Mutant E1. (48 hours) 1400x.

Figure 24. Mummified Larva with Intact Fat Body (f) and Muscle (m). Some hemocytes are present (arrows). Mutant E1. (12 days) 1400x.


s
g
h
m

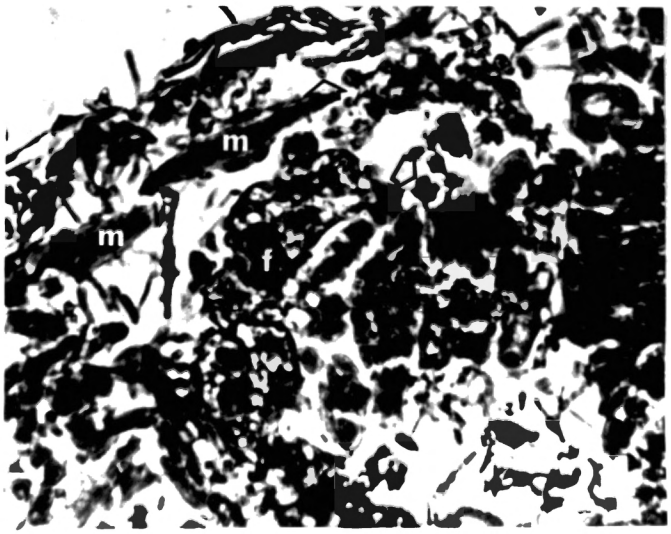

m
m
f

Figure 25. Conidia of a Low Pathogenic Mutant (#23) Unable to Germinate on the Larval Surface. (24 hours) 1300x.

Figure 26. Accumulation of Conidia Near Nodules Mutant 10. (24 hours) 1800x.


25

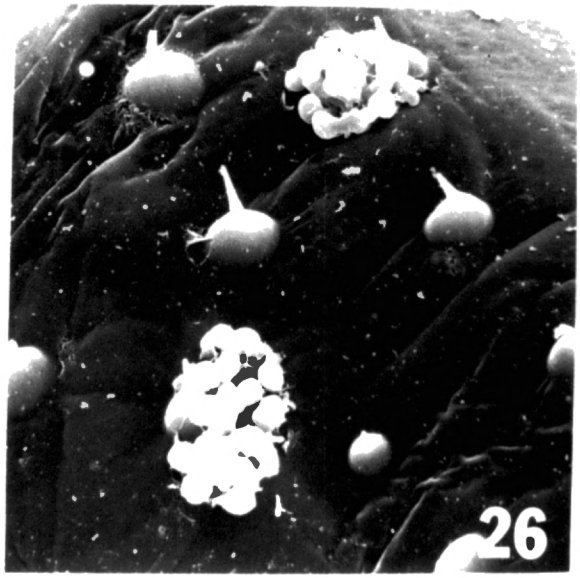

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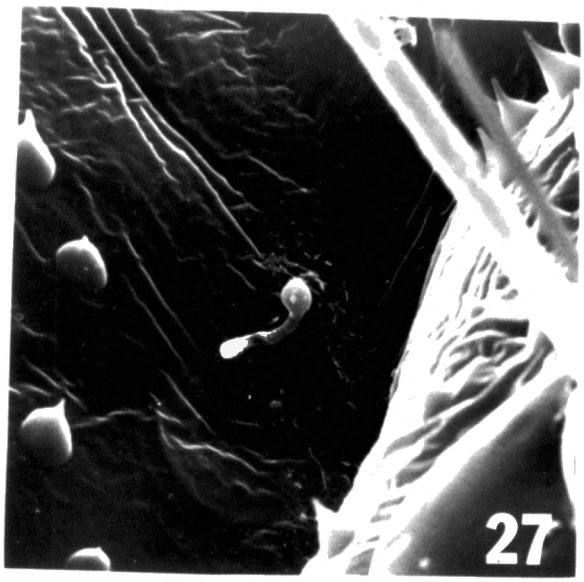
Figure 27. Isolated Conidium of Mutant 16 Producing a Germ Tube Unable to Penetrate the Cuticle. (26 hours) 2200x.

Figure 28. Hyphae of a Low Pathogenic Mutant (#9) Growing Errantly and Unable to Penetrate the Integument. (26 hours) 780x.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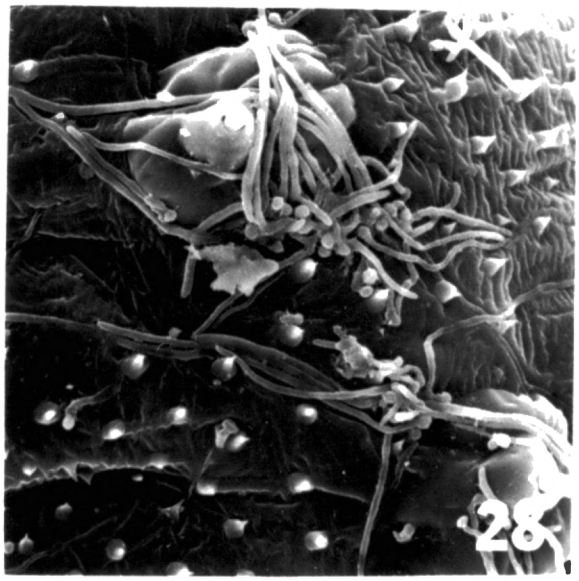
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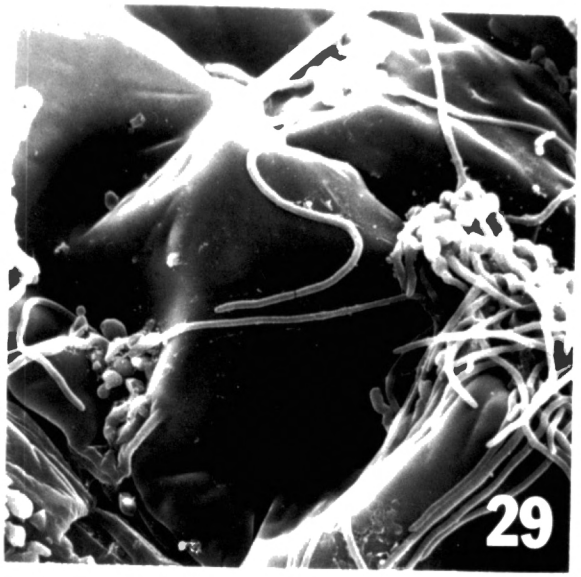
Figure 29. Errant Growth by Hyphae of a Low Pathogenic Mutant (#14). (26 hours) 1000x.

Figure 30. Errant Growth of Mutant 20 on the Larval Head. (26 hours) 480x.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30

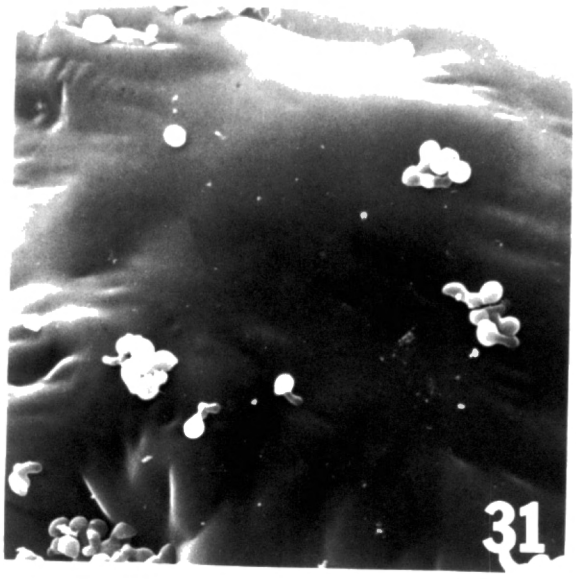
Penetration by hyphae of some poor pathogens can be observed. It can occur soon after germination (Figure 31); however, it is more commonly made by hyphae that have undergone significant elongation. The external length of hyphae may range from 2 μm (Figures 31-33) to as long as 20-30 μm (Figures 34-36). Extensive elongation of hyphae prior to penetration is rarely seen with mutant E1. As with mutant E1, when penetration does occur, it is apparently due to enzymatic activity since holes are produced at the point of entry (Figures 32-34). Appressoria are never observed.

Nonpathogenic Fungi

The conidia of fungi which are not pathogenic to the corn earworm, such as Aspergillus niger and Penicillium chrysogenum, are unable to germinate on the larval surface (Figures 37 and 38) even after 6 days. The conidia of these fungi are more uniform in shape and ornamentation.

Figure 31. Penetration by Germ Tubes of Strain R1 Soon After Germination. (26 hours) 1300x.

Figure 32. Hole Formation by a Penetrating Hypha (arrow) of Strain R1. Some growth is evident prior to penetration. (26 hours) 5800x.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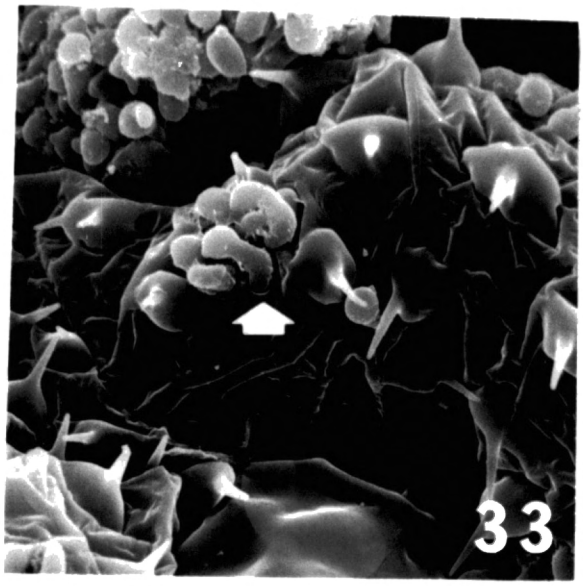
32

Figure 33. Penetration and Hole Formation (arrow) by Hypha of Strain R1. (26 hours) 2000x.

Figure 34. Penetration and Hole Formation by Greatly Elongated Hyphae of Mutant 1 (arrows). (26 hours) 1600x.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34

Figure 35. Extensive Growth and Eventual Penetration (arrow) by Mutant 14. (26 hours) 1800x.

Figure 36. Penetration (arrow) and Errant Growth by Hyphae of Mutant 20. (26 hours) 10,000x.

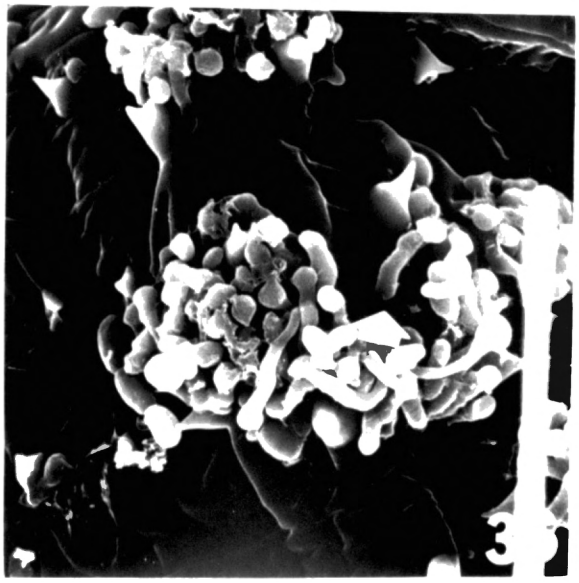
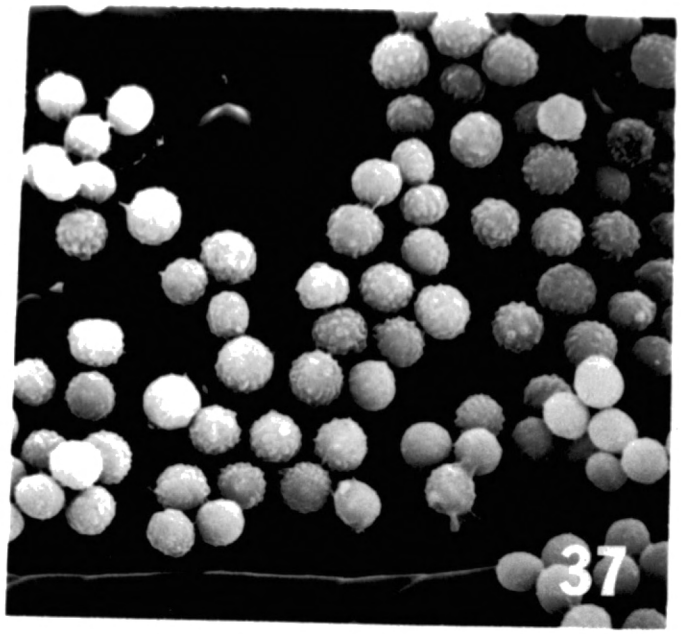


Figure 37. Nongerminating Conidia of Aspergillus niger on the Larval Surface. (6 days) 1800x.

Figure 38. Nongerminating Conidia of Penicillium chrysogenum on the Larval Surface. (6 days) 1500x.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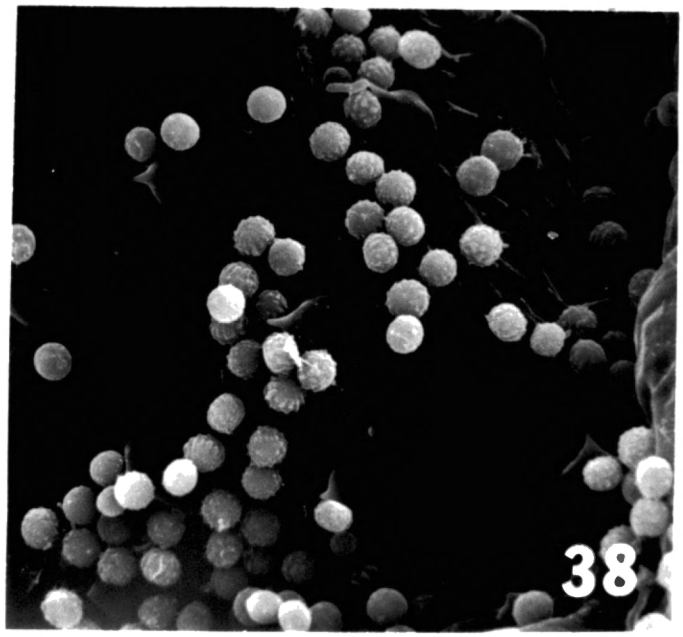
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CHAPTER IV

DISCUSSION

Since mutants of Beauveria bassiana cannot be distinguished by conidial morphology, it is assumed that no external structural feature is responsible for the variation in pathogenicity. Triton X-100 may remove some compounds from the outer wall of conidia resulting in increased ornamentation, but this does not seem to affect the behavior of conidia on the larval surface.

The consistent aggregation of conidia near setae and nodules makes one tend to consider a possible chemical interaction between conidia and cuticle. The thicker cuticle of these regions could differ from the rest of the integument in composition or deposition rate and consequently be more conducive toward conidial attachment. Of course, the mere protruding nature of these structures may be the sole reason for increased aggregation of conidia in this area. This could easily be the case since conidia adhere to all regions of the cuticle when the infection inoculum is large.

Certain strains, such as mutant 3, could have difficulty in adhering to the larval surface. However, this does not seem to be due to a morphological difference and surely does not affect the pathogenicity of this mutant.

The presence of germination on all areas of the integument negates any possible diversity in the concentration of germination activators

or inhibitors present on the cuticle. The lack of germination by Aspergillus and Penicillium may be due to inhibitors or lack of nutrients.

Following germination of conidia from all mutants of B. bassiana, three different situations have been observed: 1) immediate penetration, 2) errant growth of hyphae without penetration, and 3) a limited amount of hyphal growth with eventual penetration. The most highly rated pathogens, such as mutant E1, display immediate penetration, whereas low pathogenic mutants exhibit the two latter types of activity. Intermediate pathogenic mutants, like R1, may exhibit all three types of activity.

Holes in the cuticle at the point of penetration are usually observed and must result from enzymatic dissolution of the cuticle. These holes could not be due to wrinkling during dehydration since artifacts of that sort have an angular geometry. In all probability, wrinkling of the cuticle could not occur with the precise and uniform curvature of the observed holes. Also, shrinkage during critical point drying could not be responsible for the holes since the diameter of hyphae observed using SEM (1.5 μm) is in accordance with previously reported measurements using light microscopy (McLeod, 1954).

Since penetration by the highly pathogenic mutants occurs very soon after germination, the enzymes utilized (chitinases, lipases, proteases, and/or waxases) must be produced by the fungus during germination or soon thereafter. Poorer pathogens may require more growth before production of these enzymes can be initiated.

It is interesting to note that penetration does not occur only at the intersegmental regions as might be anticipated because of the

flexible, nonsclerotinous cuticle in these areas. Instead, penetration frequently occurs at the base of nodules. Therefore, enzymes capable of degrading hardened cuticle must be produced by the fungus.

The errant growth displayed by mutants with low infectivity is similar to that described for Metarrhizium anisopliae (Zacharuk, 1970a; Schabel, 1978). Little, if any, penetration by the very poor pathogens is observed. When it does occur, it is with the formation of holes.

The reason for such differences between mutants is unclear. It has been found that all the mutants produce enzymes capable of cuticular degradation (Grula et al., 1978). Therefore, lack of penetration cannot be due to lack of enzymes. The time of enzyme production may be important as stated above and will have to be studied. Even if this is true, some other factor(s) may also be involved. One possibility is a hyphal tip receptor which directs growth toward the cuticle. If the fungus must identify certain sites on the larval surface, it is possible that the better pathogens are able to recognize a variety of sites while poor pathogens are limited to a few; hence, the poor pathogens may need to undergo extensive growth before encountering a site.

Another possibility is that the hyphal tip is able to direct a high concentration of enzymes toward the cuticle. It may be necessary for the tip to have a semi-attachment to the cuticle before enzymes liberated there can act on the cuticle. This "focusing" mechanism may be highly regulated and more efficient in the better pathogens.

Errant growth was more noticeable for all mutants when high concentrations of conidia were used for infections. This could be indicative of a self-imposed inhibitory effect similar to that found for germination. Conidia produce germination inhibitors which prevent

germination of some conidia in large clusters. A penetration inhibitor may also be produced so that all germinating conidia in a cluster will not penetrate. The physical nature of a cluster of conidia could also interfere with the function of a hyphal tip receptor.

It is possible that infection can result from invasion through the respiratory system. As far as is known, any enzymes necessary for penetration of this system would be similar to those needed for penetration of the integument. A fatal blockage of the tracheae could result if extensive growth, without penetration, occurred.

Since the larval diet contained no antifungal agents, dislodged conidia could remain viable for some time. These conidia could then be ingested and invade via the alimentary tract. However, no evidence for this type of infection process was observed in this study. B. bassiana conidia are not visible within the gut and the gut tissue remains intact even after death and mummification have occurred. In addition, it has been observed in this laboratory that germination and growth of the fungus are extremely poor at the high pH (near pH 9) existing in the midgut.

The exact cause of death is undetermined. Apparently, soluble components of the hemolymph supply sufficient nutrients for growth and extensive multiplication of the fungus. Degradative enzymes of the fungus (lipases and proteases) are probably helpful and aid growth by solubilizing tissues such as the fat body. Due to the extensive growth of the fungus, death of the larvae could easily result from exhaustion of nutrients in the hemolymph. However, it has been found in this laboratory that minimal degradation of hemolymph proteins occurs during infection using highly proteolytic mutants. Ancillary physiological

changes, such as alterations in pH and dehydration, are most likely involved. Soluble toxins could also aid the infection by immobilizing the larvae or certain of their life systems; however, no evidence for this has been established (Champlin and Grula, 1979).

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APPENDICES

APPENDIX A

RANKING OF *BEAUVERIA BASSIANA* MUTANTS ACCORDING TO PATHOGENICITY

Selected mutants of *B. bassiana* were given the following ranking (Cheung and Grula, unpublished data) based upon probit analysis (150-200 larvae/test) of the number of dead larvae four days after infection. At this time at least 95% of those larvae which would die of the initial infection have been killed.

TABLE I

PATHOGENICITY OF MUTANTS

MUTANT	LD_{50} spores/larva	LC_{50} spores/ml
E1	15	6.31×10^4
3	30	1.78×10^5
7	35-40	6.31×10^5
16	37-40	6.61×10^5
23	70-80	5.01×10^5
1	75-80	6.31×10^5
R1	105	3.47×10^6
10	150-160	2.63×10^6
20	300	1.32×10^6
14	320	8.90×10^7
9	500	1×10^7

APPENDIX B

DEHYDRATION AND EMBEDDING PROCEDURE

Samples were dehydrated in stock solutions of tertiary butyl alcohol and ethanol in proportions outlined in Table II.

TABLE II

STOCK SOLUTIONS OF DEHYDRATION SERIES

Solution	Water	Ethanol	Tertiary Butanol
A	15	50	35
B	5	40	55
C	0	20	75
D	0	0	100

Samples washed in 70% ethanol were dehydrated and embedded in paraffin by immersion in the following sequence of solutions:

(1) Solution A for 70 minutes

(2) Solution B for 70 minutes

(3) Solution C for 70 minutes

(4) Solution D for 70 minutes

(5) Solution D overnight

(6) Solution D for 70 minutes in a vacuum

(7) Solution D with a few paraffin chips overnight (stoppered)

(8) Addition of paraffin to solution in step (7). Increase temperature to 40°C for 24 hours (unstoppered).

(9) Addition of more paraffin and further increase in temperature at 58-60°C for 2-4 hours (unstoppered).

(10) Pour off remaining alcohol and add more paraffin. Keep temperature at 58-60°C for 1 hour (unstoppered). Repeat until no alcohol is present.

(11) Embed in melted paraffin.

APPENDIX C

MODIFICATION OF HAMM'S STAIN

This stain is a modification of Hamm's stain (Hamm, 1966) for detection of virus inclusion bodies. The solutions used are as follows:

Solution A –

distilled water	100 ml
phosphotungstic acid	1.0 g
aniline blue (aqueous)	0.1 g
Orange G	0.5 g
fast green FCF	0.2 g

Solution B –

distilled water	100 ml
alizarine red S	0.1 g
glacial acetic acid	2 ml

Sections mounted on glass slides were stained with the following sequence of solutions:

(1) 2 changes of xylene for 5 minutes each

(2) 100% ethanol for 5 minutes

(3) 95% ethanol for 5 minutes

(4) 70% ethanol for 5 minutes

(5) 50% ethanol for 5 minutes

(6) Water for 5 minutes

(7) 50% acetic acid for 5 minutes

(8) Water for 2 minutes

(9) Solution A for 15 minutes

(10) Water for 5 seconds

(11) 50% ethanol for 30 seconds

(12) Water for 5 seconds

(13) Solution B for 15 minutes

(14) 50% ethanol for 10 seconds

(15) 2 changes of 100% ethanol for 30 seconds each

(16) 2 changes of xylene for 5 minutes each

(17) Mount coverslips with Eukit

APPENDIX D

LACTOPHENOL COTTON BLUE AND HEMALUM STAIN

Lactophenol cotton blue aids in detection of fungi in sections of larvae stained with Mayer's hemalum (Grimstone and Skae, 1976). Staining solutions are as follows:

Lactophenol cotton blue –	
lactic acid	20 g
phenol crystals	20 g
glycerin	40 g
water	20 ml
cotton blue	0.05 g
Mayer's hemalum Solution A –	
hematoxylin	1.0 g
sodium iodate	0.2 g
potassium alum	50 g
water	1000 ml
Shake until blue-violet, then add	
chloral hydrate	50 g
citric acid	1.0 g
Shake until red-violet	
Mayer's hemalum Solution B –	
Eosin Y saturated in 90% ethanol	

Sections mounted on glass slides were stained with the following sequence of solutions:

(1) 2 changes of xylene for 5 minutes each

(2) 100% ethanol for 5 minutes

(3) 95% ethanol for 5 minutes

(4) 70% ethanol for 5 minutes

(5) 50% ethanol for 5 minutes

(6) Water for 5 minutes

(7) Lactophenol cotton blue for 5 minutes

(8) Water for a few minutes

(9) Solution A for 2 minutes

(10) Wash with water until blue (about 10 minutes)

(11) 50% ethanol for 5 minutes

(12) 70% ethanol for 5 minutes

(13) 90% ethanol for 5 minutes

(14) Solution B for 5 minutes

(15) 100% ethanol for 5 minutes

(16) 2 changes of xylene for 5 minutes each

(17) Mount coverslips with Eukit

VITA

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Candidate for the Degree of

Master of Science

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