Protoplast Development and Histopathology of
Entomophaga grylli (Fresenius) Batko Pathotype 2
infections in Melanoplus differentialis (Thomas)

by

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Introduction and Literature Review

Pathogenic fungi of insects are potentially an important component in microbial and integrated pest management systems. Most of these fungi are thought to be environmentally safe and naturally decrease insect pest populations. Many of the entomopathogenic fungi are quite host specific, so that beneficial insect species are not affected. Insect control by fungi is also compatible with other measures, such as the use of insect resistant plants.

Although entomopathogenic fungi have these advantages, there are various problems associated with their manipulation within an agroecosystem. Some of these problems are: requirements of high humidity or free moisture, proper temperature and day-length, difficulty in mass production and storage, variation in virulence, slow acting or long infection period, proper placement, and time of release of organisms.

The above list indicates the need for basic research on entomopathogenic fungi. Additional information on fungal mode of action might suggest appropriate characteristics to select for when developing virulent strains. Research on fungal genetics and the use of biotechnology could aid in selecting fungi with greater efficacy and less stringent environmental requirements.

Taxonomic Status

Entomophaga grylli (Fresenius) Batko is a Zygomycete in the order Entomophthorales and family Entomophthoraceae (Humber, 1984). _Entomophaga grylli_ is specific to grasshoppers (Humber, 1984) and has been separated into two pathotypes (Soper et al., 1983). Two to three additional pathotypes have been recently proposed (Ramoska, unpublished data).

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The two original pathotype designations were based on isozyme analysis (Soper et al., 1983), but they also have other differentiating characteristics. Pathotype 1 produces external conidia or internal resting spores, epizootics occur in humid environments, and the host range includes Dissosteira, Camnula, Locusta, and occasionally Melanoplus species. Pathotype 2, the object of this study, lacks the external conidial cycle but produces resting spores internally. It can occur in dry environments and infects only Melanoplus species. The degree of difference between the two pathotypes suggests two different species (Soper et al., 1983). Evidence for additional pathotypes is based on host infectivity patterns (Ramoska, unpublished data). These additional pathotypes all produce external conidia. Discussion in this study which refers to a particular pathotype will be stated as such.

Entomophaga grylli possesses many characteristics in common with Entomophaga aulicae (=Entomophthora egressa), but the host range for E. aulicae is lepidopterous larvae. Although thought to be the same species by many investigators, Humber (1984) concluded that E. aulicae is a separate species using isozyme analysis.

Life Cycle

The life cycle of E. grylli pathotypes is believed to be the same except as noted. The pathotype 2 life cycle is summarized in Figure 1. The occurrence of karyogamy (the union of nuclei) and meiosis in the resting spore was proposed by McCabe et al. (1984) but has not been confirmed.

Conidia are the infective stage of the fungus and enter by cuticular invasion (Krueger and Ramoska, 1985). After reaching the grasshopper hemocoel, hyphae are thought to release protoplasts (wall-less cells) into the hemolymph. The first mention in the literature of

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protoplasts in the natural life cycle of E. grylli was by Soper et al. (1983). Protoplasts were used for their isozyme analysis, some of which were obtained from live (moribund) grasshoppers. Previous to this, E. grylli protoplasts had been obtained by germinating conidia in culture (MacLoed et al., 1980). The protoplasts multiply in the hemocoel and eventually produce a cell wall. These walled cells, called hyphal bodies, are typically oblong-shaped.

In external conidia-producing forms (e.g. pathotype 1), the hyphal bodies elongate, penetrate the integument, and form conidiophores, which in turn produce pyriform conidia (Skaife, 1925). Occasionally resting spores are produced.

In pathotype 2 the hyphal bodies produce globose immature (nondormant) resting spores. When mature, the resting spore has a thick cell wall and is dormant. Resting spores take three or more days to germinate in water, and then produce germ conidia when provided with the proper conditions. Most pathotype 2 infected grasshoppers produce resting spores only, but pathotype 2 is also capable of producing conidia (Humber and Ramoska, 1986). Conidia are formed from hyphal bodies and immature resting spores when exposed to atmospheric oxygen concentrations. These infective conidia are thought to be naturally produced when the grasshopper dies, then its body softens, the cuticle ruptures, and the hemocoel contents "stream" onto surrounding substrates. The conidia are designated cryptoconidia since they are formed at sites temporally and spatially removed from where conidia are typically found (Humber and Ramoska, 1986).

Some Disease Characteristics

Infected grasshoppers are behaviorally similar to healthy ones until just prior to death. Rate of consumption, fecal production, and activity levels are not decreased for infected grasshoppers (Ramoska,

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unpublished data). Grasshoppers infected with E. grylli have a characteristic behavior just prior to death. Dying grasshoppers climb toward the top of surrounding vegetation, where they clasp on to the plant just prior to death. Death usually occurs 12-14 days after inoculation (MacLeod et al., 1980). Naturally occurring epizootics of E. grylli are devastating when conditions are optimal (Pickford and Riegert, 1964). Some grasshoppers which have died from an E. grylli infection become very flaccid and soft internally, which results in the cuticle rupture and "streaming" referred to by Humber and Ramoska (1986).

Entomophthoralean Infections
Penetration

The conidia that contact host cuticle adhere with a mucilaginous coat (Charnley, 1984). Brey et al. (1986) concluded that the mucous coating of Conidiobolus obscurus is initially fluid but then changes to a plastic-like substance. Brobyn and Wilding (1983) suggested that this substance is protoplasm which envelopes the conidia at the time of discharge, while Eilenberg et al. (1986) concluded that the substance in Entomophthora spp. originated from between cell wall layers. Conidial germ tubes of C. obscurus penetrate the pea aphid directly (within 20 um) or grow errantly over the surface of the cuticle (Brey et al., 1986). The germ tubes of Entomophthora muscae penetrate both sclerites and the softer intersegmental membrane of its fly host (Brobyn and Wilding, 1983).

A variety of structures are used to penetrate the insect host integument. Erynia blunckii germ tubes penetrate the cuticle with a simple tube, an enlarged tip, or a screw-type structure (Tomiyama and Aoki, 1982). A thick germ tube, without extra swelling, is used by

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Conidiobolus apiculatus (=Entomophthora apiculata) to penetrate the host cuticle (Lambiase and Yendol, 1977). A combination of physical and chemical processes are used by these structures to penetrate the host cuticle (Zacharuk, 1973; Charnley, 1984). Pathogenic fungi apparently use lipolytic, chitinolytic, and proteolytic enzymes to penetrate the cuticle (Gabriel, 1968; Charnley, 1984).

Protoplast

Protoplasts occur in the in vivo life cycle of at least 6 genera of Entomophthoraceae: Entomophaga, Entomophthora, Erynia, Massospora, Neozygites, and Strongwellsea (Humber, 1984; Butt et al., 1981). Some proposed advantages of why protoplasts form in the host insect are: efficient feeding through pinocytosis, efficient reproduction without the formation of a cell wall, ability to overcome host defense by rapid multiplication (Butt et al., 1981), and superior ability to escape host detection over walled cells (Humber, 1975). The natural occurrence of protoplasts also makes mass culture and biochemical techniques easier (Murrin et al., 1986; Nolan, 1985).

For those species which have protoplasts in the natural life cycle, both in vivo and in vitro studies are important. Although the in vitro environment is better for experimental manipulation, unforeseen changes can occur. For example, morphological differences are found between E. aulicae protoplasts grown in vivo and those grown in vitro (Tyrrell, 1977). Protoplasts from infected larvae are larger, less regular in shape and exist in single units, while those grown in culture are uniform in shape and grow in chains of spindle-shaped cells.

Studies of E. grylli pathotype 1 protoplasts grown in vitro have shown that optimal growth occurs at 6.7 pH, 30°C, and 325-350 mosmols/kg (Dunphy and Chadwick, 1984). This optimum osmolarity

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correlates with the host hemolymph osmolarity. In vitro pathotype 2 protoplasts grow more slowly than those of pathotype 1 (Soper et al., 1983).

The protoplasts of E. aulicae have been studied extensively in vitro (reviewed by Nolan, 1985). These studies include morphogenisis and regeneration of protoplasts, effects of physical and chemical factors on growth, description and comparison of two distinct physiological races, and interaction with insect hemocytes.

One characteristic of Entomophthoralean protoplasts is their mobility during part of their life cycle. Protoplast movement was first observed in the 1880's by Sorokin (as cited by Tyrrell, 1977). Sorokin observed Entomophthora colorata amoeba-like protoplasts making slow but discernable movements. The protoplasts were able to change their shape and travel across the microscope slide. Entomophaga aulicae protoplasts grown in vivo have one or more tapering processes in constant motion (Tyrrell, 1977).

Several researchers have noted the lack of an immune response to protoplasts or hyphal bodies (Humber, 1975; Brobyn and Wilding, 1977; Dunphy and Nolan, 1980; Brey et al., 1986). Possible methods of adaptation to the host include having chemically nonreactive surfaces, incorporation of host biochemicals onto the cell surface, or molecular mimicry (Humber, 1975; Dunphy and Nolan, 1980).

Studies of E. aulicae protoplasts have shown that surface biochemicals are one factor involved in this lack of an immune response. Some experimentally induced surface changes result in an increased immune response. Exposure of protoplasts to papain (Dunphy and Nolan, 1981) or galactosidase (Dunphy and Chadwick, 1985) results in an increase in hemocyte adhesion or a decrease in virulence respectively, suggesting the role of carbohydrates or glycoproteins as

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critical surface components. Other surface factors implicated are hemocyte-protoplast electrostatic repulsion (Dunphy and Nolan, 1980) and absence of cell wall components (chitin or its monomer, N-acetyl-glucosamine) (Dunphy and Nolan, 1982a). There is also experimental evidence of protoplast defensive mechanisms. An increase in protoplast density correlates with a decrease in hemocyte density (Dunphy and Chadwick, 1985). Possible reasons for this decrease in hemocyte density were hemocytes adhering to host tissues, hemocyte lysing, or hemocyte clumping. Granule discharge (Dunphy and Nolan, 1982a), and protein secretion (Dunphy and Chadwick, 1985) may also be involved in defensive actions.

Histopathology

The histopathology of several Entomophthoralean fungal infections has been studied by Brobyn and Wilding, (1977, 1983). They found that in four species which infect aphids, the typical sequence of tissue colonization was fat body, nerve tissue, and muscle tissue (Brobyn and Wilding, 1977). The muscle was colonized at or shortly before death. The gastrointestinal tract and tracheae were not invaded. The development of Conidiobolus obscurus (=Entomophthora thaxteriana) is initially localized near the penetration point, while the development of Erynia aphidis (=Entomophthora aphidis) and Entomophthora planchoniana is more localized to the head and abdomen, respectively (Brobyn and Wilding, 1977).

The hyphal bodies of Entomophthora muscae were distributed throughout the hemocoel of its host, but they multiplied most rapidly in the abdomen (Brobyn and Wilding, 1983). Fat body was colonized and assimilated more rapidly than other tissues. Oocytes and tracheae were not damaged by the infection.

Humber (1975) studied infections of Strongwellsea magna in its fly host. Besides the formation of a sealed hole in the abdominal cuticle, the nervous system was the tissue most affected by S. magna. Hyphae penetrated the abdominal nerves, grew forward, and eventually reached the brain. Fat body and hemocytes were less extensive in infected hosts than uninfected hosts. Correlation of amount of fat body with the diseased state could not be made because fat body of the normal adult fly may dissociate. There were always some substantial patches of fat remaining in the infected fly (Humber, 1975). Hyphae were never found penetrating the fat body tissue.

Fungal Ultrastructure

Ultrastructural studies of Entomophthoraceae spp. which have protoplasts in their natural life cycle have been done by Humber (1975) on S. magna and by Butt et al. (1981) on Erynia neoaphidis.

Humber (1975) found that many ultrastructural aspects of S. magna are typical of other fungi, such as the presence of mitochondria with parallel cristae, glycogen rosettes (alpha particles), lipid droplets and dense bodies. Dense bodies seemed to be formed from the aggregation of electron dense areas of the endoplasmic reticulum (Humber, 1975).

Protoplasts of Erynia neoaphidis vary in size and shape (irregular, oval, amoeboid, or filamentous), and ultrastructural differences were noted between protoplasts and walled cells (Butt et al. 1981). Protoplasts had more rough endoplasmic reticulum and multivesicular bodies than walled cells but fewer electron dense bodies.

Mitochondria were smaller, oval, and with few cristae in protoplasts, but they were elongate, cup, or doughnut shaped with numerous cristae

in walled cells. Differences depend on the developmental stage of the
fungus, and possibly the position within the host (Butt et al. 1981).

Insect Tissue Changes (TEM)

There is a lack of information about ultrastructural changes of
host tissue caused by infections of Entomophthoraceae species. A study
of Metarrhizium anisopliae (Deuteromycete) infections in elaterid
beetles by Zacharuk (1971) reported on ultrastructural changes to host
tissues. Changes included an initial increase in lysosomes,
endoplasmic reticulum (ER), and ribosomes. This was followed by
vesiculation of the ER and the mitochondrial cristae, and vacuolation
of the cytoplasm. There was also a rapid disappearance of glycogen
granules and lipid and oil inclusions in the host tissue (Zacharuk,
1971).

Causes of death

It is likely that insect death from a mycosis is the result of
several factors (Charnley, 1984). Toxins or starvation are two
specific causes of death which are frequently cited. It has been
suggested that most of the lower fungi (including the Entomophthorales
sp.) kill their host by starvation, rather than by toxins (Roberts,
1981). A few toxin-producing Entomophthorales species have been
reported, but most of these compounds proved to be enzymes (Roberts,
1981). Enzymes were not included in Roberts' definition of a toxin. A
heat-labile toxin was reported from E. aulicae, but the compound was
not identified (Dunphy and Nolan, 1982b).

The objective of this study is to give an account of the
histopathology of E. grylli pathotype 2 infections in Melanoplus
differentialis, and to study the development of the fungus and its
interaction with the host.

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Materials and Methods

Adult Melanoplus differentialis grasshoppers used throughout this study were either from a laboratory colony or field collected from the Manhattan, KS area.   The laboratory colony was initially obtained from Dr. J. E. Henry (USDA Rangeland Insect Laboratory, Montana State University, Bozeman, MT 59717), and has been maintained at Kansas State University since February 1985.   Grasshoppers were reared on leaf lettuce and/or rye grass and bran, in cages measuring 30 cm x 30 cm x 45 cm.   Bran was supplemented with sulfonamides to minimize protozoan infections (Henry and Oma, 1975).

Entomophaga grylli pathotype 2 spores were obtained either from Dr. B. McDaniel (Dept. of Plant Science, South Dakota State University, Brookings, SD 57007), or were field collected in the Manhattan, KS area.   Inoculations for TEM and light microscopy (LM) studies were initiated by dipping the grasshoppers into a suspension of hyphal bodies and immature resting spores obtained from the hemocoel of a dead, E. grylli infected grasshopper.   Inoculated grasshoppers were retained in a plastic moist chamber for 24 hours.

Hemolymph Smears

Protoplast observations were made by drawing hemolymph through the abdominal intersegmental membrane using a drawn glass micropipette. Hemolymph samples were examined using phase contrast, dark field, or Nomarski interference optics.

Light Microscope (LM) Methods

Samples were taken on 4, 6, 8, 10, and 13 days post inoculation (DPI), and 12 and 36 hours post mortem (HPM). Individual tissues were

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dissected due to the difficulty in sectioning the hard exoskeleton of an intact grasshopper. Five tissues were dissected: fat body (consisting of loose sheets in the hemocoel), reproductive system (the male system in all but the 10 DPI sample), gastrointestinal (GI) tract, central nervous system (CNS), and muscle. Male grasshoppers were taken for all samples except for the 10 DPI sample. Preliminary studies showed that the infection was associated with fat body. Males were preferentially chosen because fat body was associated with the testes but not with the female reproductive system. Also adult females were frequently gravid which would alter the physiology of the grasshopper.

The tissues were fixed in 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1M phosphate buffer. Samples were dehydrated in a graded series of ethanol, placed in a transitional fluid (toluene), then infiltrated and embedded in paraffin. The complete processing schedule is outlined in Table 1. Embedded samples were sectioned 5 to 8 μm in thickness, and then mounted on glass slides. Sections were stained in hematoxylin and buffered eosin (Table 2).

Transmission Electron Microscope (TEM) Methods

Samples for TEM were taken from the same grasshoppers as for LM samples. Small pieces of tissue were excised and fixed in the same solution as for LM methods, followed by fixation in osmium tetroxide (Table 3). Tissue samples were dehydrated in a graded acetone series, then they were infiltrated and embedded in Spurr's resin (Spurr, 1969). Silver and gold sections were cut with glass knives and mounted on Formvar-coated 200 mesh thin-bar grids. Sections were stained in uranyl acetate and lead citrate and examined on a Phillips 201 electron microscope at 60 kV.

Scanning Electron Microscope (SEM) Methods

Fungal penetration was studied using the SEM. The grasshoppers were exposed to the fungus using a conidial shower. The conidial shower consisted of hemocoel contents of a freshly dead grasshopper (including hyphal bodies and immature resting spores) spread on a water agar dish. This dish was inverted over uninfected grasshoppers (separated by a wire mesh) inside a moist chamber. Microscope coverslips were also placed under the conidial shower to monitor conidial production and germination. Grasshoppers were taken out of the conidial shower at 24 hours, frozen at -20 C, then freeze-dried for 24 hours. Sections of the grasshoppers were mounted on SEM stubs and sputter-coated with gold and palladium. Samples were viewed on an ETEC Autoscan at 20 kV.

Table 1. Processing schedule used to fix and embed _M. differentialis_ tissues for LM.

1. fixative: ....1 hr
       1.5% paraformaldehyde w/v
       1.5% glutaraldehyde v/v
       0.1M PO_4 buffer

2. fixative change ....24 hrs

3. 70% ethanol v/v ....15 to 60 min

4. 80% ethanol ....15 to 60 min

5. 90% ethanol ....15 to 60 min

6. 95% ethanol ....15 to 60 min

7. 100% ethanol with 0.1% eosin ....15 to 30 min

8. 100% ethanol (2 changes) ....15 to 30 min ea.

9. toluene (2 changes) ....15 to 30 min ea.

10. toluene:paraffin (1:1) 60 C ....12 to 24 hrs

11. paraffin (2-3 changes) 60 C ....12 to 24 hrs

12. embed in paraffin

Table 2. Hematoxylin and buffered eosin staining schedule[1] used to prepare sections for LM.

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1. toluene (2 changes) ....2 min ea.

2. 100% ethanol (2 changes) ....1 min ea.

3. 95% ethanol v/v (2 changes) ....1 min ea.

4. 70% ethanol ....1 min

5. distilled water ....1 min

6. Harris hematoxylin ....2 min

7. wash in tap water ....30-60 sec

8. blue in base (1.5% $NaHCO_3$ w/v) ....30-60 sec

9. wash in tap water ....30-60 sec

10. differentiate in acid alcohol[2] ....5 sec

11. wash in tap water ....30-60 sec

12. blue in base ....30-60 sec

13. wash in tap water ....30-60 sec

14. buffered eosin[3] ....20 sec

15. wash in water ....30-60 sec

16. 95% ethanol ....30-60 sec

17. 100% ethanol ....1 min

18. toluene (2 changes) ....2 min ea.

19. attach cover slip

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[1]Moore, Brian. 1982. Solution to Eosin Staining Problems. Histo-Logic: Technical Bulletin for Histotechnology 12:185. Lee G. Luna, ed. Lab-Tek Division, Miles Laboratories, Inc. 39 W 475 North Aurora Rd. Naperville, IL 60566

[2]acid alcohol: add 10 ml 12.1M HCl to 1000 ml 95% ethanol

[3]buffered eosin:
Solution one: add 5.75 ml glacial acetic acid to 1000 ml H_2O
Solution two: add 8.2 gm sodium acetate to 1000 ml H_2O
Mix 295 ml of solution one with 705 ml of solution two. Add 5 g of eosin (C.I. No. 45380).

Table 3.   Processing schedule used to fix and embed <u>M.</u>
<u>differentialis</u> tissues for TEM.

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 1. fixative: ....1 hr
       1.5% paraformaldehyde w/v
       1.5% glutaraldehyde v/v
       0.1M PO_4 buffer

 2. fixative change ....1 hr

 3. 0.2M PO_4 Buffer rinse (3 changes) ....15 min ea.

 4. OsO_4 in 0.1M buffer (2 changes) ....30 min ea.

 5. distilled water (4 changes) ....5-10 min ea.

 6. 30% acetone v/v ....5-10 min

 7. 45% acetone ....5-10 min

 8. 60% acetone ....5-10 min

 9. 75% acetone ....5-10 min

10. 90% acetone ....5-10 min

11. 95% acetone ....5-10 min

12. 100% acetone (3 changes) ....5-10 min

13. acetone:Spurr's (1:1) ....overnight

14. 100% Spurr's (2-3 changes) ....4-12 hrs ea.

15. Embed at 70 C ....14-18 hrs

Penetration (SEM)

Numerous conidia were found attached to the grasshopper cuticle with a mucous substance (Fig. 2). The mucous material encircled the conidia at the point of attachment to the cuticle and formed a layer separate from the cell wall. Conidia were examined on both sclerites and intersegmental membrane, but conidial germ tubes were not found penetrating the host or growing on either surface. The conidia may have penetrated directly into the host, obscuring the penetration tube. In contrast, most conidia on the coverslip under the same conidial shower had germinated at 24 hours.

Upon penetration into the host, the germ tube was thought to release a protoplast directly into the hemocoel. No evidence of hyphal development was found within the hemocoel in the following studies.

Protoplast Development and Pathogenesis (LM)

Protoplast development was divided into three stages, but change is gradual and therefore transitional forms exist. These stages are proposed in order to aid in description of the disease process. Protoplasts at different stages of development can exit in a grasshopper at one time.

Amoeboid Protoplast Stage (1-7 DPI)

A low density of protoplasts was first found in hemolymph four days post inoculation (DPI). Prior to the fourth day, the infection was either small or localized and could not be identified. The protoplasts at this stage were amoeboid in shape, capable of movement, and were designated amoeboid protoplasts. Amoeboid protoplasts were colorless, had fibrillar extensions (pseudopodia), and usually lacked

refractile granules in the cytoplasm (Fig. 3). Protoplasts are multinucleate, but the nuclei were indistinct at this stage.

Movement of the protoplasts occurred primarily during the amoeboid protoplast stage. Time-lapse micrographs (Figs. 4-9) show protoplast movement consisted of slow side to side swaying of part of the cell, and swelling and contracting. Also, some protoplasts had fibrillar pseudopodia (Fig. 3) which visibly extended and retracted. Pseudopodial and protoplast movements did not typically occur simultaneously, and neither motion resulted in movement across the slide.

Developing Protoplast Stage (6-10 DPI)

Developing protoplasts became rounded, and lost their amoeboid shape and movement (Fig. 3). Refractile granules became visible, but protoplasts remained colorless. The nuclei were indistinct as in the amoeboid protoplast stage. The protoplast density in the hemolymph increased greatly by 6 DPI.

Mature Protoplast Stage (8-14 DPI)

The mature protoplasts were round, brown in color in transmitted light, and contained numerous refractile granules (Fig. 10). The nuclei were very visible in some protoplasts (Fig. 10), but became obscured by the numerous granules in others (Fig. 11). The protoplast density in the hemolymph increased during the 8-14 DPI period which caused the hemolymph to become a turbid white color rather than the normal yellow. Also, during this period the hemocyte density decreased, and granules similar to those in the protoplasts were found in the hemolymph.

Immune Response to Protoplasts

Hemolymph smears were examined for a cellular immune response to protoplasts. Many grasshopper hemolymph samples were checked, but samples from only one grasshopper showed a positive cellular immune response. Hemolymph samples taken 10 DPI showed both phagocytosis and the beginning stages of encapsulation taking place (Figs. 12 and 13). Phagocytized protoplasts were found in several stages of digestion by the hemocytes. Protoplasts which had been engulfed for some time, consisted of aggregations of refractile granules and cytoplasm (Fig. 14). The grasshopper was checked again at 19 DPI, but no protoplasts were found. The immune response of this grasshopper may have eliminated the infection.

Hyphal Body and Immature Resting Spore

Hyphal bodies were formed from mature protoplasts by producing a cell wall at approximately the time of host death. No visual identification of a walled cell was made in live hosts. The cell wall gradually increased in width, and eventually a visible cell wall was formed on protoplasts. Once the cell wall was formed, the structure was called a hyphal body (Fig. 15). Hyphal bodies , elongated (Fig. 16), germinated (Fig. 17), or formed immature resting spores (Fig. 18). The elongated and germinated hyphal bodies were found most frequently in the leg joints or below other areas of intersegmental membranes. Conidia were occasionally found on the exterior of grasshoppers where breaks in the integument had occurred. The most common development sequence observed was hyphal body, immature resting spore, thick walled (mature) resting spore (Fig. 19).

Histopathology (LM)

Only sections of control, early infection (6 DPI), late infection (13 DPI), and post mortem (12 HPM) samples are shown, since progression of the disease is slow. Very few protoplasts were found in sections of tissue from the 4 DPI sample, so sections from 6 DPI were used as the early sample. The late infection CNS tissue sample was from 10 DPI.

Control tissues were sampled in order to determine changes which were disease related. Uninfected grasshoppers had large deposits of fat body in the form of unattached sheets distributed in the hemocoel. Fat body also surrounded and was attached to other tissues in the hemocoel (see below). Control fat body was composed of highly vacuolated trophocyte cells which had no apparent cell separation between nuclei (Fig. 20).

All of the reproductive tract samples shown in Figures 21, 27, 32, and 37 are testes (see Materials and Methods for explanation). The control testes consisted of a series of testicular follicles. Sperm were found within each follicle at various stages of development (Fig. 21). A fat body layer was located around the testes and between follicles.

The GI tract was divided into distinct regions (stomodeum, mesenteron, proctodeum), but each region was composed of epithelial cells surrounded by muscle (Fig. 22). The amount of muscle surrounding the GI tract varied according to the region where the sample was taken. Malpighian tubules were attached to the GI tract and were frequently found lying next to it.

The CNS was composed of segmental ganglia with ventral longitudinal connectives between them. Ganglia had a central area, the neuropile, which contained nerve fiber tracts. The neuropile was

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surrounded by glial cells and nerve cell bodies, which were enclosed by neural lamella (Fig. 23). Fat body was frequently attached to the CNS.

Muscle tissue occurred as skeletal muscle throughout the thorax and abdomen (Fig. 24). Some of the muscle tissue had fat attached. Trachea were associated with all tissues.

Early Infection Tissue (6 DPI)

Protoplasts were found predominantly on fat body tissue, both on unattached sheets of fat as well as on fat body associated with other tissues (Fig. 25). The E. grylli infection on 6 DPI was localized. Some sections of fat body had many protoplasts attached, while others had none or only a few (Figs. 25 and 26). Occasionally, protoplasts were found within the fat body, but serial sections revealed that protoplasts found within the fat body had grown through natural openings. There was evidence that some protoplasts grew along tracheae within fat body. Protoplasts were attached to fat body around the testes and between follicles. They remained in contact with fat body and did not penetrate the follicles (Fig. 27). Protoplasts were attached to fat body that surrounded the CNS, as well as within the ganglia, penetrating the neuropile (Fig. 29). These protoplasts were typically hypha-shaped. Determination of how the fungus entered the nervous system was not made. No infection was found in the connectives. Only occasionally were protoplasts attached to GI tract or skeletal muscle tissue (Figs. 28 and 30). Tracheae and air sacs occasionally had protoplasts attached.

Various protoplast shapes were found. Most of the protoplasts were round to oblong (developing protoplasts, Fig. 25), but some were thread-like (also developing protoplasts, Fig. 26). Occasionally, amoeboid protoplasts were found on 6 DPI.

Late Infection Tissue (13 DPI)

Protoplasts were noticeably larger, more rounded in shape, and formed a sheath that covered most surface area of the fat body sheets at 13 DPI (Fig. 31). Although fat body was the primary site of colonization, some fat body remained at the end of the infection period. Fat body of the testes was also completely covered with protoplasts, and it appeared somewhat reduced in volume between follicles (Fig. 32). Protoplasts had increased in density within the ganglia by 10 DPI, and continued to penetrate the neuropile. The cell body region was unaffected. Occasionally protoplasts were found within connectives. Protoplasts within the CNS were still hypha-shaped (Fig. 39). Sections of the brain from 10 DPI revealed fungal penetration there as well. Some protoplasts were found attached to parts of the GI tract and muscle, but not as consistently or as concentrated as on fat body (Figs. 38 and 40).

Post Mortem Tissue (12 HPM)

Fat body had degraded considerably and only remnants could be identified following death (Fig. 36). Likewise, the fat body surrounding the testes and follicles was digested, although not the follicles themselves. The surface of the follicles was often depressed by the presence of hyphal bodies (Fig. 37). The infection was again found within the CNS, with some of the cells having a rounded or hyphal body shape (Fig. 39). The infection was again centered in the neuropile area, but no common areas of invasion were found in the neuropile between samples. Occasionally, hyphal bodies were found in the connectives. The GI tract had a few hyphal bodies penetrating into the surrounding muscle, but none were found in the lumen of the gut (Fig. 38). Hyphal bodies were frequently found penetrating the skeletal

muscle after death (Fig. 40). Occasionally these hyphal bodies were elongated.

Protoplast Development and Ultrastructure

Preliminary investigation revealed that protoplasts were associated with fat body, therefore, this tissue and the protoplasts attached to it were studied using TEM. The same categories of protoplast development were used as in the LM study.

Few amoeboid protoplasts were attached to fat body at this stage. Those which were attached had only a small surface area in contact with the fat body (Fig. 41). Pseudopodia were numerous on some protoplasts (Fig. 42). Mitochondria and nuclei were the most common organelles. Small dense bodies were prevalent, but very few lipid droplets were found (Fig. 43).

Developing protoplasts became rounded, lacked pseudopodia, and had more surface area in contact with fat body than amoeboid protoplasts (Compare Figures 41 and 44). Some protoplasts became completely flattened against the fat body (Fig. 45). Dense bodies had enlarged and the protoplasts began to accumulate lipid droplets (the refractile granules seen under light microscopy).

The mature protoplasts were also rounded in shape and occurred in dense numbers, surrounding the fat body (Fig. 46). Lipid droplets and dense bodies filled the cells.

The cell membrane of the amoeboid protoplast was irregular and was not very electron dense compared to later stages (Fig. 43). Some early protoplasts had a diffuse membrane which may have been due to the cutting angle. Vesicles formed from invaginations of the cell membrane were found in some of the amoeboid cells (Fig. 41). As the protoplasts matured the cell membrane became more dense and regular in shape. Few cell membrane invaginations were found in developing

protoplasts except for cell division septa (Fig. 44). These septa were almost exclusively found in the developing protoplasts. Mature protoplast membrane was electron dense, and areas adjacent to fat body frequently had small invaginations (Fig. 47). Protoplasts often had a visible cell coat (glycocalyx) (Figure 47).

Nuclei of E. grylli were large, had one or two nucleoli, and contained a large amount of heterochromatin. Amoeboid protoplast nucleoli were larger, more irregular in shape, and less electron dense than the nucleoli of later stages (compare Figures 48 and 49). Most nuclei were in the interphase stage of mitotic division. Occasionally a metaphase nuclei was found (Fig. 44).

Mitochondria were of various shapes (Figs. 43 and 47), with surprisingly few cristae. Many of the mitochondria were swollen and did not seem to be well preserved. The problem may have been due to low osmolarity of the fixative or buffer rinses. Attempts to correct the problem by adding sucrose to the fixative and buffer were unsuccessful.

Dense bodies were numerous in the amoeboid protoplasts, but small in size. The largest amoeboid dense body vesicle was 0.9 μm, while the dense bodies of developing and mature protoplasts were as large as 1.5 μm.

The morphology of the dense bodies changed as the protoplasts developed. The dense material was confined to a small area within a larger vesicle in the amoeboid protoplasts; in the developing protoplasts the dense material filled the entire vesicle but was often granular in appearance. Most of the dense bodies in the mature protoplasts were completely electron dense, although a few had a granular appearance.

Very few lipid droplets were found in the early amoeboid protoplasts (Fig. 50). More were found in developing protoplasts (Fig.

51), while mature protoplasts were filled with droplets (Fig. 52).
There was also an increase in lipid droplet size. The few droplets
found in the amoeboid protoplasts ranged in size from 0.3 μm to 0.6 μm
(Fig. 50), while those in the mature protoplasts were as large as 2.3
μm (Fig. 52).

Many of the mature protoplasts had large deposits of glycogen
particles (Fig. 47). Glycogen particles were not found in samples of
earlier stages. The protoplast cytoplasm had free ribosomes and
polysomes (Fig. 43), but no positive identification was made of
endoplasmic reticulum or Golgi cisternae.

Hyphal body and Immature Resting Spore Ultrastructure

Hyphal body and immature resting spore ultrastructure was similar
to mature protoplast ultrastructure except for the addition of the cell
wall. Cell walls of various thicknesses were found in both the 12 and
36 HPM samples. Thicker cell walls were of a single layer, uniformly
translucent, and usually had a smooth even surface (Fig. 53). Thinner
cell walls were not as electron translucent, and had a mottled
appearance (Fig. 54).

The cell wall of a forming hypha was thicker at the base than on
the tip (Fig. 55). The thin cell wall was diffuse (Fig. 56). Numerous
small dense bodies were located toward the tip, but there were no large
lipid droplets at the tip of a newly formed hypha produced from a
hyphal body.

Insect Ultrastructure

Control fat body had large lipid droplets, deposits of glycogen
particles, and abundant rough endoplasmic reticulum and mitochondria.
Large deposits of glycogen particles were often adjacent to lipid

droplets (Fig. 57). The cytoplasm was light in density and organelles were easily identifiable.

The infected fat body (13 DPI) also had large lipid droplets, but the cytoplasm was much more dense and therefore more difficult to resolve organelles (Fig. 58). Mitochondria and ER were present. No glycogen particle deposits were found, as in the control fat body.

Discussion

Penetration (SEM)

The mucous coat surrounding the conidia aided in attachment to the host, but it appeared to be separate from the conidial cell wall. Thus it probably originated from the conidiophore (possibly protoplasm), rather than from between cell wall layers as occurs in the genus Entomophthora (Eilenberg et al., 1986).

Although no penetrating structure of E. grylli was found, it is possible that penetration took place directly below the conidia. The presence of germinated conidia on the coverslip under the same conidial shower indicated that conidia were viable and that conditions were adequate for germination.

Protoplast Development

The protoplast developmental sequence indicates a change in function as well as in shape and content of the protoplasts. Amoeboid protoplasts circulated in the hemolymph and were usually not in contact with fat body; their function appeared to be dispersal. Developing protoplasts increased in density and began to cover the fat body surface. Cell division septa were found most frequently in developing protoplasts indicating a higher rate of division. The developing protoplast function appeared to be reproduction and colonization of the fat body. The mature protoplasts increased in size and accumulated light refractile granules (lipid droplets). Their primary function appeared to be the sequestering of sufficient nutrients before host death.

Development of E. aulicae protoplasts in vitro has been described by Dunphy and Nolan (1977b). Numerous stages of development were

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described, and although not all of these stages may occur _in vivo_, some similarities with E. grylli development were found. Both species had protoplasts that exhibited pseudopodia movement at one stage and cells (protoplasts for E. grylli and hyphal bodies for E. aulicae) which became more granular with age.

Other Entomophthorales spp. may follow a similar protoplast developmental sequence, but since most have a shorter infection period, the stages may pass quickly. Butt et al. (1981) also noted that protoplast changes depended on the developmental stage, but he did not correlate specific changes with stages of development.

Protoplast Movement

Pseudopodia and protoplast movements probably aided in locomotion, but they did not typically occur simultaneously for E. grylli. Protoplasts did not move across the slide as Sorokin observed for Entomophthora colorata (as cited by Tyrrell, 1977). Similarly, one stage of E. aulicae protoplasts had pseudopodia which moved, but the protoplasts did not move in the media (Dunphy and Nolan, 1977b). Actin filaments are present in fungal cells and probably play a role in cell and pseudopodial movement in E. grylli protoplasts.

Host Immune Response

A cellular immune response to E. grylli was uncommon. Other investigators have found similar results. Brobyn and Wilding (1977) did not observe an immune response in four Entomophthora spp. infecting aphids. Neither a cellular nor a humoral immune response to C. obscurus was found in the pea aphid (Brey et al., 1986). Phagocytosis is typically thought to occur to small particles, while large particles are encapsulated, so the presence of large phagocytized protoplasts was surprising. The observed immune response to E. grylli protoplasts may

have been due to a biochemical surface change as studies on E. aulicae indicated (Dunphy and Nolan, 1981; Dunphy and Chadwick, 1985), or a superior immune system of the grasshopper. The decrease in hemocyte density towards the end of the infection period suggested immune suppression by the fungus.

Hyphal Body and Immature Resting Spore

Formation of a cell wall at approximately the time of host death would be advantageous. The internal environment of the host changes rapidly after death, making the hemocoel an unsuitable place for growth. Cell wall formation may have indicated a depletion of nutrients. In vitro studies of related species have shown that cell wall formation (Dunphy and Nolan, 1977a) and sporulation (Latge, 1980) occur when the amount of nutrients in the media were depleted.

The elongated hyphal bodies below membrane areas were similar in shape and location to those in pathotype 1 which penetrate the cuticle and produce conidia. Their presence below intersegmental membrane suggested an attempt by E. grylli pathotype 2 to penetrate the integument and produce external conidia. Germinated hyphal bodies were thought to serve a similar function as elongated hyphal bodies, i.e. produce conidiophores which in turn produce conidia. The occurrence of the modified hyphal bodies below membrane areas may correspond to greater oxygen permeability of the membrane than the sclerites. Humber and Ramoska (1986) reported that conidia production occurred in an environment of increased oxygen concentration. The elongation of hyphal bodies may also be cued by oxygen concentration.

Although conidia were occasionally formed when the cuticle broke, the most common sequence was protoplast, hyphal body, immature resting

spore, and resting spore. The fungal development of E. grylli is summarized in Fig. 59.

Histopathology

The tissues most affected by E. grylli were similar to that found by Brobyn and Wilding (1977); fat body, CNS, and muscle. Interaction between protoplasts and insect tissue involved both adhesion and penetration.

Fat body, both unattached sheets and that attached to other tissues, was the preferred site of protoplast attachment, beginning before 6 DPI. Protoplasts were occasionally attached to other tissues, but at much lower densities. The infection was localized on 6 DPI, but the sampling procedure did not allow observation of where the infection was more prevalent in the hemocoel. Attachment of protoplasts primarily to fat body probably corresponded to the high concentration of energy and nutrients found there. Reduced fat body volume (especially between follicles) indicated nutrients were being used by the protoplasts, but there was always some fat body remaining at the end of the infection. Only remnants of the fat were found after host death. The degraded condition of the fat body after death was probably due to natural metabolic breakdown as well as digestion by the protoplasts and hyphal bodies.

Penetration of protoplasts within the CNS was in progress by 6 DPI and was concentrated in the ganglia throughout the infection period. Samples were not taken in a way that would indicate progression of the disease through the CNS, but very few protoplasts were found in the connectives between ganglia. Thus it appeared that the fungus entered the CNS at various locations and did not move forward through the CNS as observed by Humber (1975). Presence of fungi in the CNS may have

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influenced the climbing behavior prior to death, and may have been a contributing factor in host death.

The muscle was penetrated by the fungus, but not until after host death. After death, the follicles were deformed by the presence of hyphal bodies. This change may have been caused either by metabolism by the hyphal bodies or loss of cell integrity by the follicles following death. The GI tract muscle was invaded, but not the lumen. Tissue changes which were observed only post mortem were not thought to be significant in the cause of death.

Interpretation of the micrographs must take into consideration that tissues were taken out of their natural setting in the hemocoel. Most of the protoplasts grew and circulated in the hemolymph, particularly during the early stage of the infection, and the tissue samples could not account for those protoplasts. When a grasshopper died, the hemocoel was packed with hyphal bodies, many of which were not attached to tissue.

The most significant fungus-tissue interaction probably took place before host death. The formation of a cell wall, around the time of host death, would slow uptake of nutrients. Thus, the fungus caused little additional damage to the tissue. While some nutritional uptake may have taken place after host death and cell wall formation, most of the cells were either preparing for a resting stage, or attempting to reach the exterior (elongated hyphal bodies). The change in tissue shape and structure after host death was at least partly due to loss of tissue integrity. Histopathological changes caused by the E. grylli infection are summarized in Fig. 59.

Protoplast Development and Ultrastructure

The ultrastructural study of pathotype 2 protoplasts revealed that changes in shape corresponded with changes in ultrastructural detail.

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The membrane changed from irregular in shape in the amoeboid protoplast to more regular in shape in later stages. Most cell division septa were found in developing protoplasts, which indicated a higher rate of division in that stage. Mature protoplast membrane had small invaginations in some areas. These irregularities of the membrane suggested pinocytosis was taking place. Butt et al. (1981) also observed invaginations on Erynia neoaphidis which suggested pinocytosis.

The large nuclei contained great amounts of heterochromatin. This is typical for other Entomophthoraceae spp. and has been correlated with a large genome size for E. aulicae (Murrin et al., 1986). The genome size is more than 100X larger than most other fungi. A large amount of heterochromatin usually occurs in prophase of mitosis. Large amounts of heterochromatin were also found in Erynia neoaphidis by Butt and Beckett (1984). They hypothesized that most nuclei were in prophase and the interphase mitotic stage did not exist. Humber (1975) proposed that nuclei in this condition had perpetually-condensed interphasic chromosomes. Apparently, either the interphase stage did not exist or the chromosomes were condensed during interphase. Since the size of a nucleolus reflects its activity (Alberts et al., 1983), the large nucleoli in amoeboid protoplasts of E. grylli indicated a high rate of RNA production in this early stage.

Protoplast mitochondria had very few cristae and appeared swollen: this seemed to be a fixation problem. Studies by Humber (1975) of S. magna and Butt et al. (1981) of Erynia neoaphidis protoplasts also found mitochondria with few cristae. Humber (1975) concluded that this was at least partly due to poor fixation. Perhaps the mitochondria of at least some protoplasts naturally have fewer cristae.

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Dense bodies are commonly observed in fungi, although the term refers to organelles which are thought to be variable in content and function (Garrison et al., 1975; Lambiase and Yendol, 1977). Using electron cytochemical techniques, Garrison et al. (1975) found that the dense bodies (or microbody-like organelles) contained enzymes and were possibly involved in lipid metabolism. Others have suggested that either dense bodies serve as packages of protein used for spore germination (Humber, 1975) or enzymes used for host penetration (Lambiase and Yendol, 1977).

Entomophaga grylli dense bodies appeared to change from 1) a small amount of condensed material within a larger vesicle, to 2) granular material filling the entire vesicle, to 3) a completely dense material. There was also a corresponding increase in dense body size as the protoplasts matured. Humber (1975) described dense body development in S. magna and suggested dense bodies were formed from aggregations of electron opaque endoplasmic reticulum material. Evidence regarding initial formation of dense bodies in E. grylli was inconclusive. Lambiase and Yendol (1977) found that dense bodies in E. apiculata contained crystalloid inclusions. No such inclusions were found in E. grylli protoplasts.

Lipid droplets are commonly found in Entomophthorales spp. (Humber, 1975; Lambiase and Yendol, 1977). The lipid content of one Entomophthorales sp., Conidiobolus coronatus (=Entomophthora coronata), was as high as 44 percent (Mumma and Bruszewski, 1970). Lipids consisted of both saturated and unsaturated fatty acids. For pathotype 2, the number and size of the lipid droplets increased as the protoplasts matured. Glycogen particles also became apparent in mature protoplasts. These are common in Entomophthorales spp., such as S. magna (Humber, 1975), and C. apiculatus (Lambiase and Yendol, 1977).

The accumulated lipid droplets and glycogen particles provided a source of energy for subsequent stages of development (dormancy or conidial formation).

Neither Golgi bodies nor ER were found in E. grylli. Beckett et al. (1974) stated that no Zygomycotina fungi possess Golgi bodies. Lambiase and Yendol (1977) found that C. apiculatus contained free ribosomes but not ER, as observed in E. grylli. Endoplasmic reticulum was found in S. magna by Humber (1975).

Hyphal Body and Immature Resting Spore Ultrastructure

The early cell wall appeared thin and diffuse. Later the cell wall became thicker and more electron transparent. The hyphal body and immature resting spore cell walls consisted of a single layer. Although the ultrastructure of mature resting spores was not examined, they were thought to have more than one cell wall layer as is the case for Conidiobolus obscurus. (Latge et al., 1982).

The function of the small dense bodies found at the tip of a germinating hyphal body is not known. Humber (1975) found similar "dense material" at the apex of a growing spore of S. magna. The small dense bodies may have been involved in cell wall synthesis.

Insect Ultrastructure and Cause of Death

The most significant change that occurred in fat body during the infection period was the disappearance of glycogen particles. Glycogen particles also disappeared in insect tissue infected with M. anisopliae, a Deuteromycete (Zacharuk, 1971). Reduction of glycogen particles has been found to occur during periods of reduced feeding or starvation in insects (Chapman, 1982; Dean et al., 1985). The absence of glycogen particles in the E. grylli infected tissue indicated that nutrient depletion was occurring. The moribund grasshopper continued

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to feed normally until death (Ramoska, unpublished data), but the nutrients from the GI tract may not have reached the fat body when the hemocoel was full of protoplasts.

The fat body of a healthy male insect has large lipid and glycogen supplies (Dean et al., 1985), but interpretation of fat body changes are given cautiously due to its dynamic nature. The fat body ultrastructure can be variable between different regions within an insect (Dean et al., 1985).

There were probably several contributing factors to the grasshopper's death besides nutrient depletion or starvation. The presence of protoplasts in the hemolymph would probably disrupt other physiological processes besides the transport of nutrients (e.g. immune system, hormone transport). The presence of the fungus in the CNS may have contributed to the death of the grasshopper and altered its behavior, especially with respect to the characteristic "death climb." Other observations possibly related to the cause of death include the presence of refractile granules in the hemolymph which may contain toxins and the disappearance of hemocytes by the end of the infection.

Some additional areas of research that could be pursued are: the analysis of hemolymph throughout the infection period (e.g. changes in protein, carbohydrate, or hormone levels); factors concerning cellular immune response; and effect of the fungus on the CNS, including the grasshopper's behavior.

Fig. 1. Life cycle of _Entomophaga grylli_ pathotype 2.

germ
conidium

conidium

protoplast

grasshopper
hemocoel

hyphal
body

cryptoconidia

meiosis?

cuticle
rupture

immature
resting
spore

karyogamy?

mature
resting
spore
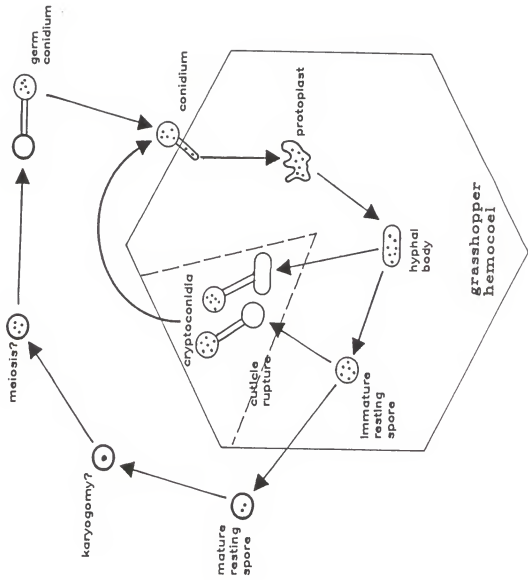
Fig. 2. Conidia (C) of *E. grylli* pathotype 2 attached to the grasshopper cuticle with a mucous-like substance (arrow). (SEM).

Fig. 3. Amoeboid (AP) and developing (DP) protoplasts. Amoeboid protoplasts typically have pseudopodia (ps) extended and no visible granules in the cytoplasm. Developing protoplasts are rounded in shape and usually have a few visible granules (gr). (Nomarski Interference).

Figs. 4-9 Time-lapse photographic sequence of protoplasts (P) showing movement. Time lapse between frames was 1-2 minutes. (Phase Contrast).

Fig. 10. Mature protoplasts (MP) filled with granules and with apparent nuclei (arrows). (Phase Contrast).

Fig. 11. Nuclei not as apparent in this mature protoplast (MP). Note the granules (arrow) that were found in the hemolymph during late stages of the infection. (Phase Contrast)
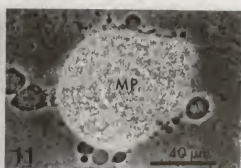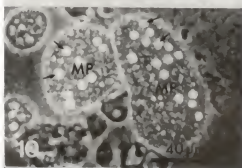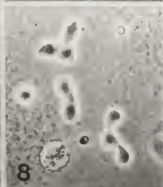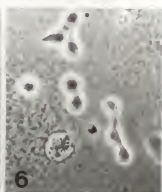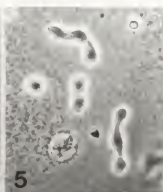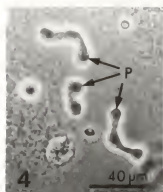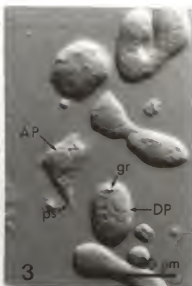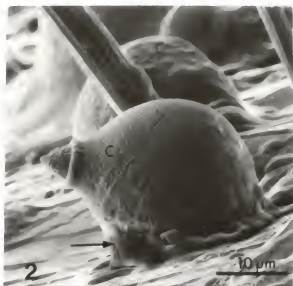
Fig. 12. Protoplast (P) phagocytized by a hemocyte (hm). (Phase Contrast). Figures 12-14 are shown at the same magnification.

Fig. 13. Protoplasts (P) being encapsulated by hemocytes (hm). (Phase Contrast).

Fig. 14. Two phagocytized protoplasts (P). One is more digested (star) than the other. Hemocyte (hm). (Phase Contrast).

Fig. 15. Hyphal bodies (hb) are of various shapes and have a cell wall (arrow). (Phase Contrast).

Fig. 16. Elongated hyphal bodies (hb) found below are of intersegmental membrane. (Nomarski Interference).

Fig. 17. Germinated hyphal bodies (hb) which are also found below intersegmental membrane. (Phase Contrast).

Fig. 18. A hyphal body (hb) forming an immature resting spore (i). (Phase Contrast).

Fig. 19. Mature resting spores (rs) have a thick cell wall. (Phase Contrast).
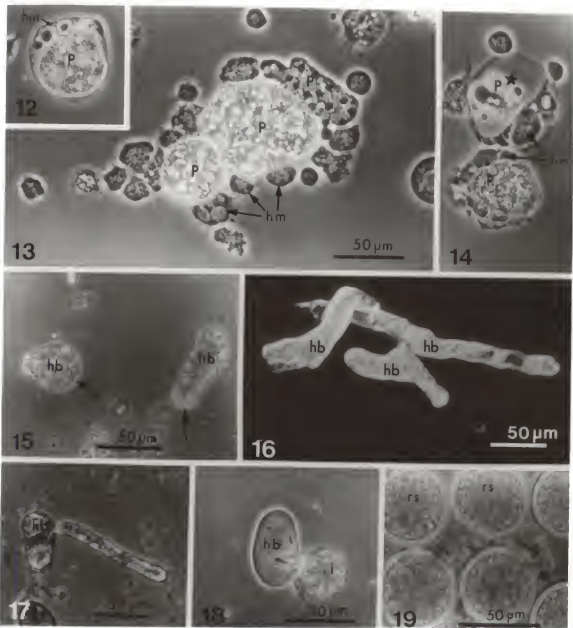
Figs. 20-24. Control tissues of M. differentialis.
(Bright field). Figures 20-30 are shown at the same
magnification.
    20. Fat Body (FB) with vacuoles.
    21. Testes with follicles (F).
    22. GI Tract. Epithelium (e), gut lumen (lu), muscle
        (ms).
    23. Ganglion (G) of the CNS. Glial cells (gc),
        neural lamella (nl), neuropile (np).
    24. Muscle (ms). Nucleus (N), trachea (tr).

Figs. 25-30. Infected tissues of M. differentialis
at 6 DPI (Days Post Inoculation). (Bright field).
   25-26. Fat Body (FB). Some areas of fat body had
        many protoplasts attached (Fig. 25); some
        had only a few (Fig. 26).
    27. Testes. Protoplasts (P) attached to the
        surrounding fat body (FB). Follicles (F).
    28. GI Tract. Epithelium (e), gut lumen (lu),
        muscle (ms).
    29. CNS. Note protoplasts (P) within the
        neuropile (np). Cell bodies (CB).
    30. Muscle (ms).
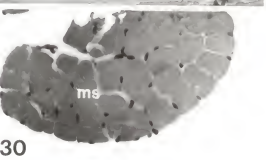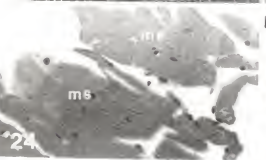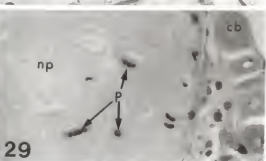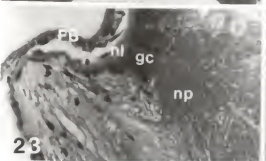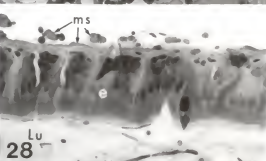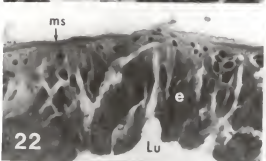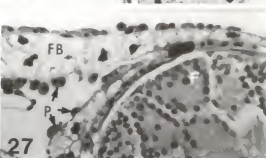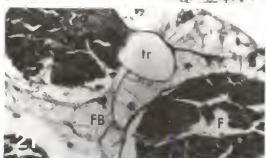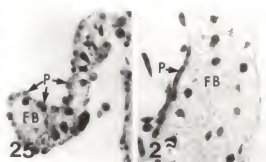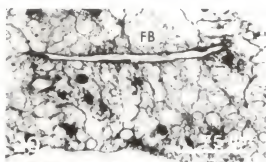
Figs. 31-35. Infected tissues of M. differentialis at 13 DPI. (CNS tissue is from 10 DPI.) (Bright field). Figures 31-40 are shown at the same magnification.

31. Fat body (FB) with protoplasts (P) attached.
32. Testes. Protoplast (P) attached to fat body which surrounds the testes. Follicles (F).
33. GI tract. Protoplasts (P) attached to trachea (tr) between the circular muscle (ms) and epithelium (e) of the GI tract. Gut lumen (lu), Malpighian tubules (mt).
34. CNS. Note protoplasts (P) throughout the neuropile (np). Cell body (cb), fiber tract (ft).
35. Muscle. A few protoplasts (P) were found attached to the muscle (ms).

Figs. 36-40. Infected tissues of M. differentialis at 12 HPM (Hours Post Mortem). (Bright field).

36. Fat Body. Hyphal bodies (hb) attached to remnants of fat body (FB).
37. Testes. Follicle (F) shape was changed by the attached hyphal bodies (hb).
38. GI Tract. Hyphal body (hb) penetrating the circular muscle of the GI tract.
39. CNS. Hyphal bodies (hb) within the neuropile (np) of a ganglion. Glial cell (gc).
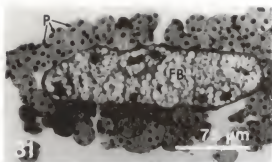40. Muscle. Elongated hyphal bodies (hb) within the muscle (ms).
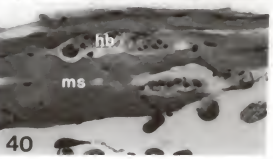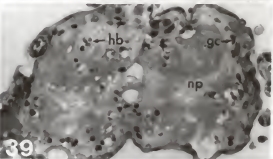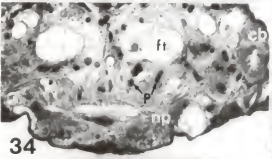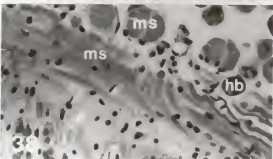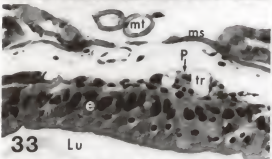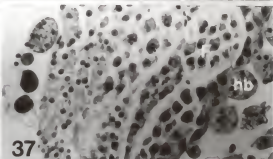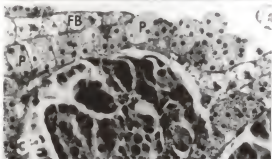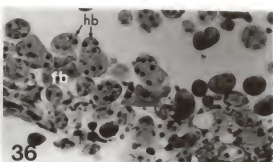
Fig. 41. Amoeboid protoplast (AP) on 4 DPI with a pseudopodia (large arrows) extended. Fat body (FB), mitochondria (mi), nucleus (N), vesicle (vs).

Fig. 42. Amoeboid protoplast (AP) on 4 DPI with numerous small pseudopodia (ps). Fat body (FB), tracheae (tr).

Fig. 43. Inset from Figure 41 shows irregular membrane (arrow), mitochondria (mi), and dense bodies (db) of the amoeboid protoplast (AP). Fat body (FB), ribosomes (circle).

Fig. 44. Developing protoplasts (DP) on 6 DPI showing cell division septa (arrows) and a nucleus (N) in metaphase. Dense bodies (db), fat body (FB), lipid droplets (L).

Fig. 45. A developing protoplast (DP) on 6 DPI with a flattened configuration. Fat body (FB), lipid droplets (L), nucleus (N).
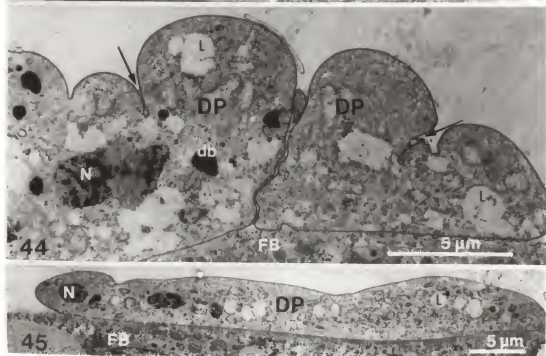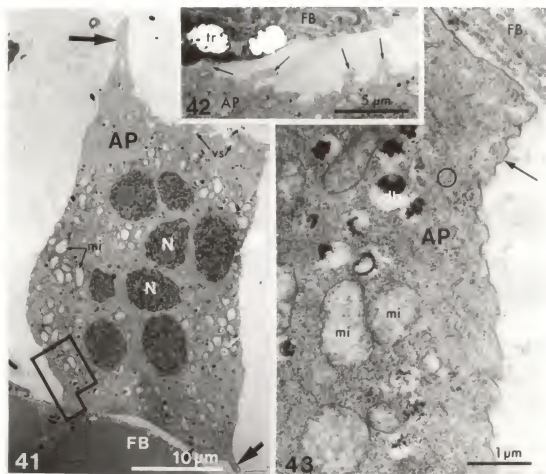
Fig. 46. A mature protoplast (MP) on 10 DPI positioned between pieces of fat body (FB). Note the numerous lipid droplets (L), and dense bodies (db).

Fig. 47. Higher magnification of a mature protoplast (MP) on 13 DPI. Note the irregular membrane (m) which suggests pinocytosis was taking place. Fat body (FB), glycogen (gl), lipid droplets (L), mitochondria (mi).
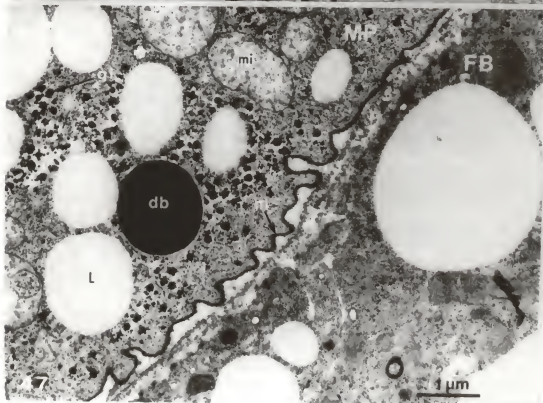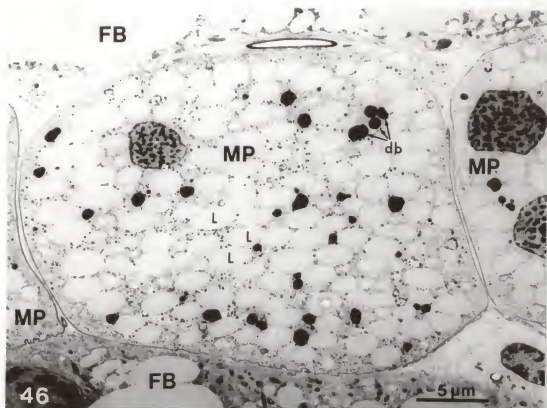
FB

MP

db

MP

L
L
L

MP

FB

46

5 µm

gl

mi

MP

FB

db

ni

L

47

1 µm

48

Fig. 48. Nucleus (N) of an amoeboid protoplast (AP) (4 DPI) with a large nucleolus (nu).

Fig. 49. Nucleus (N) of a mature protoplast (MP) on 13 DPI with a small nucleolus (nu).
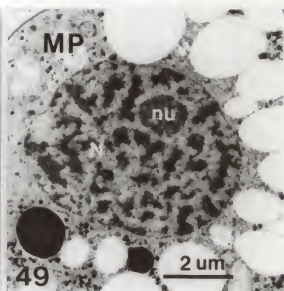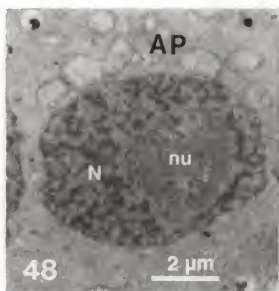
AP | **MP**

48   2 µm   49   2 um

50

Fig. 50. Lipid droplets (L) and dense bodies (db) of an amoeboid protoplast (AP). The lipid droplets were very uncommon at this stage. 4 DPI.

Fig. 51. Lipid droplet (L) and dense bodies (db) of a developing protoplast (DP). 6 DPI.

Fig. 52. Lipid droplets (L) and dense bodies (db) of a mature protoplast (MP). 13 DPI.

Fig. 53. Thick, translucent cell wall (CW) of a spore at 12 HPM. Dense bodies (db), lipid droplets (L).

Fig. 54. Thin cell wall (arrow) forming on a spore at 36 HPM.

Fig. 55. A hypha (H) growing from a hyphal body at 12 HPM. Note the small dense bodies (circle). The cell wall is thicker near the base (large arrow) than at the tip (small arrow). Lipid droplets (L).

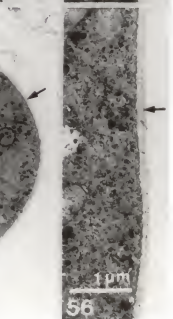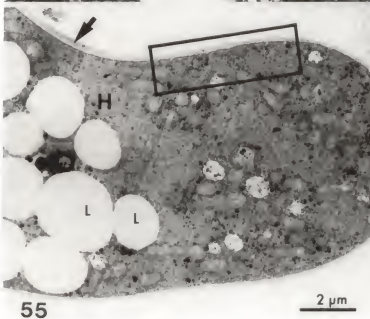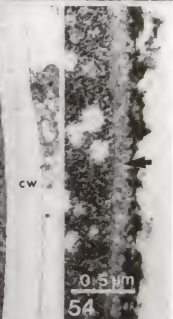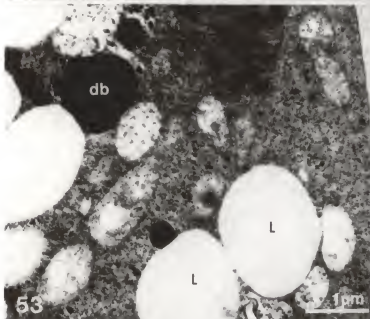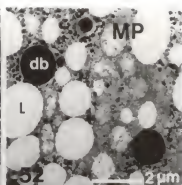Fig. 56. Inset from Fig. 55 showing the diffuse cell wall (arrow).
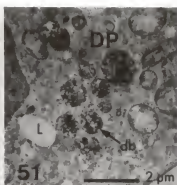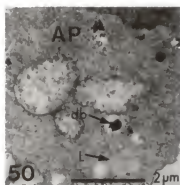
Fig. 57.   Control fat body (FB). Note the large deposit of glycogen (gl) particles and the lipid droplets (L). Endoplasmic reticulum (er), mitochondria (mi).

Fig. 58.   Infected fat body (FB) at 13 DPI.   Note the presence of the lipid droplets (L) and the absence of glycogen   particles.   Endoplasmic   reticulum   (er), mitochondria (mi), protoplast (P).

57

58

54
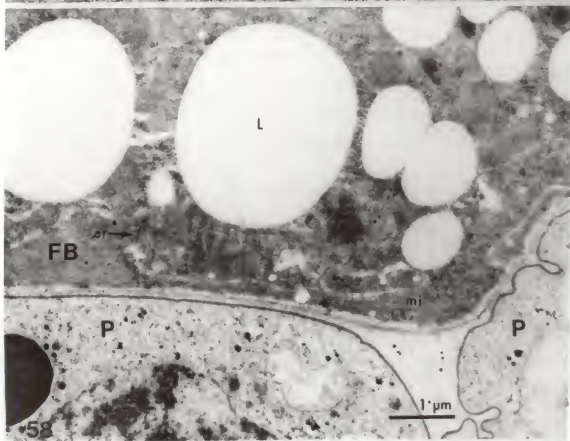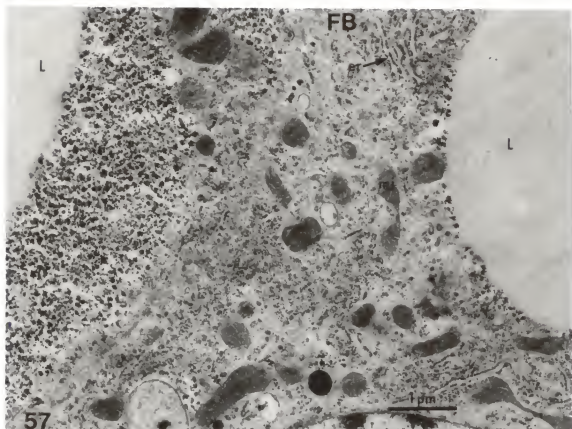
Fig. 59. Summary of fungus development and histopatholo-
gical changes during E. grylli pathotype 2 infections in M.
differentialis.

PATHOGENESIS OF ENTOMOPHAGA GRYLLI PATHOTYPE 2

	DAYS POST INOCULATION							DEATH	HOURS POST MORTEM			
	2	4	6	8	10	12	14		24	48	72	96
FUNGUS DEVELOPMENT	Amoeboid Protoplast	Developing Protoplast		Mature Protoplast					Hyphal Body	Immature Resting Spore	Resting Spore	
HISTOPATHOLOGY			Protoplasts attached to fat body	Protoplasts penetrated CNS		Protoplasts attached to muscle			Fat body destroyed	Hyphal bodies penetrated muscle		

– – – – – = Proposed

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Protoplast Development and Histopathology of
Entomophaga grylli (Fresenius) Batko Pathotype 2
Infections in Melanoplus differentialis (Thomas)

by

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B.A., Tabor College, 1982

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

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1987

Abstract

The pathogenesis of _Entomophaga grylli_ (Fresenius) Batko pathotype 2 infections in _Melanoplus differentialis_ (Thomas) was studied using light and electron microscopy. The fungus grew as a protoplast _in vivo,_ and protoplast development and ultrastructure was divided into three stages: amoeboid, developing, and mature protoplasts. Each stage was characterized by shape and ultrastructural changes. The amoeboid protoplast was amoeba-like in shape and movement, with very few lipid droplets found in the cytoplasm. Mature protoplasts were rounded, larger in size, and filled with lipid droplets. Developing protoplasts were a transitional stage between the two.

The histopathology of the five grasshopper tissues was studied: fat body, male reproductive system, gastrointestinal tract, central nervous system, and muscle. Histological sections of infected tissue revealed that the fungus attached earlier and more extensively to the fat body than to other tissues. The ganglia of the central nervous system (CNS) was extensively invaded by the fungus before host death occurred. After death, muscle tissue was penetrated by the fungus.

The ultrastructure of control and infected grasshopper fat body was examined. The most significant change that occurred was the disappearance of glycogen particles in the infected fat body. Cause of death may be attributed to nutrient depletion, fungal penetration in the CNS, as well as disruption of physiological processes due to the growth of the fungus in the hemocoel.