PURIFICATION AND INFECTIVITY OF *ENTOMOPHAGA GRYLLI* (FRESENIUS) BATKO, PATHOTYPE 2 AGAINST *MELANOPLUS DIFFERENTIALIS* (THOMAS)

by

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B.S., Kansas State University, 1974

A THESIS

Submitted in Partial Fulfillment of the Requirements
for the Degree Master of Science

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KANSAS STATE UNIVERSITY
Manhattan, Kansas
1984

Approved:

Major Professor

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ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. William A. Ramoska, Associate Professor of Entomology, for his quidance during the course of this study. His enthusiasm and professionalism set the tone for an excellent working environment. I would also like to extend my appreciation to the members of my graduate committee, Dr. T. L. Hopkins and Dr. C. L. Kramer for their helpful assistance.

I also wish to express my gratitude to Roberto Rodriguez and Theresa Todd for their technical assistance and friendship.

Finally, I wish to acknowledge my parents and family for their support and understanding during my graduate career.

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May, 1984

INTRODUCTION

Periodic outbreaks of rangeland grasshopper populations often cause economic damage to range and croplands in the Great Plains region of North America. Chemical controls often prove unsatisfactory due to their non-selective nature and the prohibitive expense involved in treating large areas of rangeland. Therefore, the naturally occurring grasshopper pathogen, Entomophaga grylli (Fresenius) Batko, is receiving much attention for use in controlling grasshopper outbreaks.

Entomophaga grylli has a worldwide distribution with recent natural epizootics occurring among Patanga succinata (L.) in Thailand (Roffey, 1968) and among Conocerus variegatus (L.) in nigeria (Chapman and Page, 1979). Mortality among Melanoplus spp. in North America was reported by Rockwood (1950) in Oregon and by Smith (1933) in Kansas. Pickford and Riegert (1964) observed a widespread epizootic in Saskatchewan, Canada in which Camnula pellucida (Scudder) and Melanoplus bivittatus (Say) were dying in the same localities from different strains of E. grylli. Using isozyme analysis, Soper et al. (1983) confirmed the existence of two pathotypes of E. grylli which would not readily cross-infect. Pathotype 1 produced conidial or resting spore states upon the death of the host and primarily infected Bandwing grasshoppers. Pathotype 2 produced resting spores only and infected Melanoplus spp. The peculiar behavior of the dying host (Skaife, 1925) is symptomatic of both pathotypes.

Since natural outbreaks of Melanoplus populations occur

frequently in the Great Plains, basic information on the epizootiology of *E. grylli* Pathotype 2 needs to be established.

Pathotype 2 is an obligate parasite and exhibits four distinct stages in its life cycle. Resting spores, germinated resting spores, and germ conidia may exist outside the host, while hyphal bodies occur in the insect hemocoel. The work described herein was directed toward establishing the agent(s) and mode(s) of infection through purification of the various stages in the life cycle, and application of these entities in *per os* and topical treatment regimes to adult *Melanoplus differentialis* (Thomas).

MATERIALS AND METHODS

Sources of *Melanoplus differentialis* (Thomas)

Adult *M. differentialis* were collected from natural populations in North Central Kansas. The insects were quarantined for at least three weeks prior to use in bioassays. Laboratory colonies were generated from egg pods obtained from Dr. R. D. Frye (Dept. of Entomology, NDSU, Fargo, ND. 58105) and from Dr. D. A. Streett (USDA, Rangeland Insect Laboratory, Montana State University, Bozeman, MT. 59717). Grasshoppers were kept in cages 1'x 1'x 1.5'; fed lettuce or field rye, wheat bran, and provided with water. The insectary was maintained at 27^{o}C, a 14-hr. photoperiod, and 40-60% relative humidity. Bioassay grasshoppers always included the youngest and healthiest adults available.

Sources of *Entomophaga grylli* (Fresenius) Batko Pathotype 2

Resting spores of *E. grylli* Pathotype 2 were obtained from *M. differentialis* and *M. bivittatus* cadavers infected in limited, natural epizootics occurring in North Central Kansas in 1981 and 1982. Additional Pathotype 2 resting spores were obtained from Dr. R. D. Frye and Dr. B. McDaniel (Dept. of Plant Science, SDSU, Brookings SD. 57007). Isolation of resting spores from cadavers generally followed that of Nelson *et al* (1982).

Production and Purification of Life Stages

Resting spores of E. grylli were germinated in 4 ml sterile, distilled water, pH 6.7, with gentamicin sulfate in 25 cm^2 tissue culture flasks. The spores were held in an environmental chamber at 24°C and 16-hr. photoperiod until germinated resting spores produced germ tubes 50-150 um long.

Germ conidia were produced by transferring the suspension of germinated and nongerminated resting spores from each flask to separate petri plates with 1% water agar. The plates were placed in a laminar flow hood with the lids removed allowing the suspending water to evaporate. As the water film surrounding the germinated resting spores sublimed, the tips of the germ tubes began to swell. Drying was continued until marked apical swelling of the germ tubes was apparent in most of the germinated resting spores (approximately 1 hr./1 ml suspending water). The plates were then covered and placed in an incubator at 24°C for an additional 4-8 hrs. to allow completion of germ conidium development.

Purified germ conidia were obtained by isopycnic centrifugation of the contents of the agar plates in Percoll density-gradient medium (Pharmacia Fine Chemicals, Newark, NJ.). The plates were irrigated with 0.15M NaCl and the germ conidia, germinated resting spores, and residual nongerminated resting spores contained in the wash were concentrated by low-speed centrifugation. Fractions of the pellet (0.2-0.5 ml) were then layered on top of 10 ml continuous Percoll gradients contained in high-speed centrifuge tubes. The gradients were centrifuged in a Sorvall (Model RC 2-B,

Ivan Sorvall Inc., Newton, CT.) super-speed centrifuge at 30,000 rfc for 20 minutes at 4°C. Fractionation of the gradients was accomplished by pulling the banded entities from the top with pasteur pipettes. The purified germ conidia were rinsed and re-suspended in sterile, distilled water.

Purified germinated resting spores were obtained by running gradients containing only germinated resting spores and residual non-germinated resting spores from tissue culture flasks. The germ conidia production step was omitted. residual non-germinated resting spores recovered from all gradients were presumed non-viable and discarded.

Fresh resting spores were produced from injections of purified germ conidia. Germ conidia recovered from gradients were rinsed in sterile, distilled water and re-suspended in saline/glycerol (30v/70v). The suspensions were delivered through a 1.0 cc disposable syringe (26g, 3/8 needle) mounted in an Isco micro-applicator (Instrumentation Specialties Co. Inc., Lincoln, NE.). Adult Melanoplus grasshoppers were anaesthesized with CO_2 and injected through the intersegmental membrane between lateral abdominal sclerites with 5.0 ul of suspension. Dose was determined with a hemacytometer. The infected grasshoppers were reared normally until killed by the fungus, yielding fresh resting spores within the cadaver.

Hyphal bodies were also produced by injecting purified germ conidia into M. differentialis. Infected hosts were sacrificed after 7 days and their body cavities irrigated with Hanks balanced saline solution (Hanks and Wallace, 1949). The hyphal bodies contained in the wash were rinsed several

times and re-suspended in Hanks. Bioassays with the various stages in the life cycle of Pathotype 2 always employed the freshest material available.

Per os Bioassays

Per os bioassays consisted of force feeding hyphal bodies, resting spores, germinated resting spores, and germ conidia to adult M. differentialis. Purified samples of each life stage were suspended in saline/ glycerol (30v/70v) and 10 ul was delivered into the oral cavity of grasshoppers that had been starved for 24 hours. The suspensions were delivered through a 1.0 cc syringe (26g, 3/8 needle) mounted in an Isco micro-applicator. Dosage was determined with a hemacytometer. The hosts were individually caged and fed for three weeks following treatment and observed for disease development. Fecal material was collected to monitor passage of the fungal entity through the digestive tract.

Topical Bioassays

Infectivity of fresh resting spores, germinated resting spores, and germ conidia was investigated by suspending pure samples of each life stage in 2.0 ml sterile, distilled water (0.1% Tween 20, Wilding 1976) and topically applying each suspension to 28 anaesthisized, surface sterilized, M. differentialis on a stream of compressed air through a hand held atomizer. Dosage was determined from marked glass slides placed among treated hosts. Immediately following spray

treatment, the grasshoppers were transferred into individual cages and incubated for 48 hours in environmental chambers at 95% relative humidity, 24°C, and ambient room light (2-44 ft-candles). Grasshoppers were not fed during the incubation period. After 48 hours, the grasshoppers were removed from the environmental chambers and placed in the insectary (27°C, 40-60% RH). They were fed daily and observed for three weeks for disease development. Percent mortality was computed as: the number of hosts killed by the fungus divided by the total number of insects per treatment. Percent mortality was uncorrected for control mortality.

Investigation into topical germ conidium infectivity as influenced by relative humidity and light involved spray applications of the purified life stage to adult M. differentialis. Germ conidia, suspended in sterile, distilled water (0.1% Tween 20) were evenly divided into 5, 2.0 ml samples. Dosage was determined with a hemacytometer. Each suspension was delivered to 20 anaesthisized, surface sterilized hosts on a stream of compressed air through a hand held atomizer. Immediately following spray treatment, the grasshoppers were transferred into individual cages and incubated for 24 hours in environmental chambers under various relative humidity and light regimes. Twenty grasshoppers each were held under both constant light (52-90 ft-candles) and constant dark conditions at 90% and 100% relative humidities. A hand held squeeze-trigger atomizer was used to periodically mist the grasshoppers with sterile, distilled water (0.1% Tween 20) during the incubation period in treatments involving 100% constant

relative humidity. Control grasshoppers were sprayed with germ conidia that had been killed by immersion for one hour in commercial sodium hypochloite/sterile, distilled water (50v/50v). Control grasshoppers were incubated at 100% relative humidity under constant light. Temperature was kept constant at 24^{o}C for all treatments. A subsample of each conidial suspension was delivered onto a 1% water agar plate and incubated with its respective treatment. Percent germination of germ conidia on agar plates after 24 hours was determined by dividing the number of germinated germ conidia by the total number of germ conidia from 10 counts. Germination was followed with a Wild inverted phase contrast microscope. Grasshoppers were not fed during the 24 hour incubation period. After 24 hours, the grasshoppers were removed from the environmental chambers and placed in the insectary, fed daily, and observed for three weeks for disease development. Percent mortality was determined as in the previous experiment.

Scanning Electron Microscopy (SEM) Studies

Germ conidia germination on the host integument was viewed by freeze-drying whole grasshoppers that had been incubated for 8 hours at 90% and at 100% relative humidities after a spray application. Grasshoppers were quenched in iso-pentane before transfer into liquid nitrogen. Freeze-drying required 48 hours. The specimens were sectioned, then mounted onto SEM stubs with colloidal silver paste. A layer of gold and palladium was evaporated onto their surface.

Germ conidia were viewed at 20 Kv with an Autoscan SEM.

Bioassay Cages

Because of the epizootic potential of E. grylli and the gregarious and cannibalistic behavior of grasshoppers, individual cages (Fig. 1) were built to hold grasshoppers during bioassays. The contagious spread of opportunistic pathogens was also minimized by caging hosts individually during treatment and disease development.

RESULTS AND DISCUSSION

Production and Purification of Life Stages

Experimental quantities of germ conidia could be efficiently produced and purified by the methods described in this study. Experience has shown that two criteria were important for greatest yields. First, agar plates should not be seeded with more than 4×10^6 spores from the tissue culture flasks. A higher percentage of germinated resting spores were able to initiate germ conidium production on lighter seeded agar plates. As the suspending water was evaporated, the spores tended to clump, modifying the microclimate, leading to variation in the rate of germ conidium development on different areas of the plates. Lighter seeding rates are therefore important in maximum, uniform germ conidium production from the maximum number of germinated resting spores. Second, the 4-8 hour period for completion of germ conidium formation following the drying procedure needed to be monitored for the optimum time for irrigation of the life stages from the agar plates for density-gradient purification. Plates were irrigated after release of most of the germ conidia from empty germ tubes and prior to their germination. Some experience was necessary to determine when to irrigate the plates as incompletely formed germ conidia and germinated germ conidia could not be harvested with the bulk of mature germ conidia from density gradients.

The typical banding pattern for *E. grylli* Pathotype 2 resting spores, germinated resting spores, and germ conidia

in Percoll gradients made to a starting density of 1.070 g/ml is shown in Figure 2. Germinated resting spores were contained in the top band and had a buoyant density ranging from 1.040-1.050 g/ml. The top band also contained all the empty germ tubes and approximately 10-20% germ conidia. The majority of germ conidia were recovered from a diffuse band (1.055-1.085 g/ml) exhibiting considerable heterogeniety. Much of the variation in density of germ conidia could be an artifact of the drying procedure used in their production. It was common for varying small quntities of protoplasm to be left in germ tubes and not incorporated into the mature germ conidia when the duration and rate of drying was not uniform, as occurred when spores clumped on agar plates. Germ conidia contained in the top band probably contained less protoplasm or were trapped in the mat of germ tubes during centrifugation. Non-germinated resting spores were the most dense life stage (1.080-1.120 g/ml) and moved to the bottom of the gradients. The variation in density of resting spores was probably due to variation in the amount of protoplasm and in the thickness of the spore wall.

The degree of separation between bands could be manipulated by changing the starting density of gradients (Fig 3). Percoll gradients with a lower starting density increased the separation between germinated resting spores and germ conidia, while gradients with a higher starting density increased the separation between germ conidia and non-germinated resting spores. Overloading gradients with large samples tended to

decrease the separation between the top two bands, decreasing the harvest of pure germ conidia. Germ conidia purified from large samples required the centrifugation of many gradients.

Harvesting the life stages contained within gradients was accomplished by suction of the bands from the top with pasteur pipettes. Low speed centrifugation of the recovered germ conidia during rinsing with sterile, distilled water served to remove contaminants associated with the top band. Batches of germ conidia with greater than 98% purity were commonly obtained with Percoll density-gradient centrifugation. Generally, 6-8 hours were required to purify 7-10 million germ conidia from 16-20 agar plates. Purified germ conidia were stored overnight at $4^{o}C$ in sterile, distilled water and used in bioassays the following day.

Valovage _et al_. (unpublished) has reported Pathotype 2 germ conidia production from resting spores incorporated within 1.5% water agar in standard 100 mm petri plates. The resting spores germinated in the agar and produced germ conidia at the agar surface which were released over a period of 3-4 days. Grasshoppers were topically innoculated with germ conidial showers by inverting petri plates them. The technique of purifying germ conidia with density-gradients is significant in that greater flexibility in formulation and application is possible. Field trials with germ conidia will require manipulation of discreet batches of conidia. Innovative techniques in the production and purification of _E. grylli_ life stages are therefore important in establishing the potential for use of this pathogen in grasshopper manage-

ment programs.

Injections

Production of fresh cultures of resting spores was necessary during the course of this study. No artificial media has been developed to support the complete life cycle of E. grylli, so injections of some life stage into Melanoplus hosts was necessary to produce fresh resting spores. A young, adult female may contain as many as 5×10^6 resting spores. Nelson et al. (1982) obtained infections in Melanoplus hosts from injections of suspensions containing germinated and non-germinated resting spores. An elaborate stirring mechanism was required to maintain a uniform suspension. When this method was used in the present study, the inoculum tended to fall out of suspension in the collar of the syringe. Clogging occurred leading to variation in the delivered dose over a series of injections. Injections of germ conidia suspended in saline/glycerol (30v/70v) were routinely used for stock spore increases in the present investigation. Germ conidia became contorted in the hyperosmotic suspension, but remained pathogenic when injected into grasshoppers. Percent mortality of 140 field collected, adult M. differentialis injected with an average of 744 fresh germ conidia each is shown in Figure 4. Mortality due to E. grylli occurred between the 8th and 21st day. The mean time from inoculation to death was 13.9 days (SE = 0.74).

Suspensions of germ conidia in saline/glycerol (30v/70v) were easy to handle and remained uniform during the course

of a series of injections. Fresh germ conidia are more immediately viable than resting spores, capable of germination in 2-4 hours, while 1-4 days were required for germination of resting spores. Viability was generally higher with germ conidia than with resting spores. Germ conidia may provide a reliable standard for LD_{50} comparisons between injections of Pathotype 2 isolates.

Per os Bioassays

Nolan et al. (1976) suggested the possibility for per os infections with Entomophthora egressa MacLeod and Tyrrell resting spores based on the effect of pH on germination. Walton and Fenton (1939) reported 25% mortality of unidentified grasshoppers after they were provided bran baited with E. gryllı spores. In the present investigation, per os bioassay of all stages in the life cycle of Pathotype 2 failed to produce any infections in adult M. differentialis. All life stages were pathogenic when injected, but not pathogenic when ingested (Table 1). Examination of fecal material recovered from petri plates attached to the bottom of individual bioassay cages yielded dessicated, non-viable germinated resting spores and germ conidia. No intact hyphal bodies were recovered. Resting spores recovered from fecal pellets and plated onto 1% water agar showed 1-2% germination after 4 days. Therefore, it does not seem possible for the disease to be transmitted to healthy individuals by ingestion of any life stage, although dissemination of resting spores from cannibalized cadavers or from vegetation may influence epizootiology.

Topical Bioassays

Germ conidia produced from overwintering resting spores are probably responsible for establishing initial infections each season (Soper and MacLeod, 1981). Spray application of E. grylli Pathotype 2 resting spores, germinated resting spores, and germ conidia to adult M. differentialis in the present study (Table 2) supports this contention. Mortality resulting from germ conidia application was ten times higher than that with germinated resting spores. Since Pathotype 2 lacks the external conidial cycle produced when the host dies in humid conditions, germ conidia appear to be the only infective life stage in this fungus throughout the season. Tyrrell and MacLeod (1975) suggested the possibility for infection with germ tubes of Entomophthora aphidis Hoffman resting spores. Resting spore germ tubes of E. grylli Pathotype 2 appear not to be invasive. The one infection obtained with this treatment probably could be attributed to contaminating germ conidia in the treatment suspension or to the eventual production of germ conidia from the germinated resting spores lodged on the host integument. No mortality was seen with grasshoppers sprayed with Pathotype 2 resting spores. Depending on the age of the inoculum and the nature of the suspending medium, resting spores generally required from 1-4 days in saturated conditions for germination. Germination of resting spores presumably did not occur within the 2 day treatment period of the bioassay.

The percent mortality with adult M. differentialis

sprayed with germ conidia and incubated for 24 hours at 24°C under various relative humidity and light conditions is shown in Table 3. Binomial analysis showed significantly higher mortality among hosts incubated at 100% relative humidity in constant light when compared with hosts incubated at 100% relative humidity in constant dark ($0.01 < p < 0.02$). Germ conidia viability on agar plates held in continuous light and dark did not differ significantly after 24 hours within each replication. There also was no correlation between germ conidia viability and percent mortality between replications. Scanning electron micrographs (Figs. 5 and 6) of freeze-dried thoracic segments revealed germination of germ conidia at 100% relative humidity, but not at 90% relative humidity. Mortality among hosts held at 90% relative humidity suggests the possibility for infections from low dosages, presumably from germ conidia contained in water droplets trapped between sclerites. The requirement for high relative humidity for conidial germination has been demonstrated for many of the Entomophthorales (Yendol, 1968; Newman and Carner, 1975; Soper et al., 1975).

A majority of insects infected with entomopathogenic fungi die in the late afternoon. Conidiogenisis proceeds during the night with discharge of conidia occurring early the next morning (MacLeod , 1963). Subsequent infections of suitable hosts or secondary conidium production from discharged conidia, thus probably takes place during the daylight hours. Among the Entomophthorales that respond phototropically, periods of light serve to orient the organism for dispersal through aiming the conidiophore and discharge of conidia

toward the source of illumination (Page, 1965; Page and Humber, 1973). The findings in the present investigation with Pathotype 2 germ conidia appear to contradict the bulk of knowledge concerning phototropic responses with externally born conidia produced from diseased hosts. Infections with Pathotype 2 germ conidia tended to increase with grasshoppers held in constant light at high relative humidity (Table 3). This suggested a negative phototropic response indicating the formation of infective germ tubes rather than the production of secondary conidia. Although not closely related, germ tubes of some species of parasitic rust fungi are negatively phototropic (Gettkandt, 1954). Germ conidia, representing a distinct life stage, may not adhere to the phototropic pattern seen with externally produced conidia. The addition of Tween 20 to the inoculating suspension may also have influenced the light response of germ conidia. Additional investigation into phototropic responses with all stages in the life cycle of entomopathogenic fungi and their formulation is required to more fully understand their use and epizootiology.

In natural environments, germ conidia probably contact grasshoppers in two ways. Resting spores germinate off the host on saturated substrates and produce germ conidia that land on, or are picked up by passing grasshoppers. Alternatively, infections may result from germ conidia produced from the germination of resting spores carried on the host integument. In either scenerio, a saturated micro-environment is required for resting spore germination. Dew formation and conditions not conducive to excessive grasshopper activity

for several days may be sufficient to trigger resting spore germination, germ conidia production and subsequent infections. The results of this investigation indicate that a macro-environment above 90% relative humidity facilitates germ conidia infectivity possibly by enhancing the longevity of trapped water droplets in which germ conidia germination takes place.

Data on germ conidium dosage required to produce infections in Melanoplus hosts is inconclusive. As stated earlier, there was no correlation between viability on agar plates and grasshopper mortality following topical treatment of germ conidia. In natural environments, E. grylli probably exhibits a clumped dispersion about previously infected cadavers. Dosage would vary directly with proximity to centers of inoculum. Resting spore/germ conidium viability and host population density probably exert considerable influence on the likelihood of germ conidium-host contact under appropriate climatic conditions.

Epizootiology of E. grylli is probably further influenced by the condition of the host population. The percent mortality attributed to several etiological agents of over 230 older grasshoppers collected from a field population exhibiting no incidence of E. grylli is shown in Figure 7. When older grasshoppers were injected with germ conidia for stock spore increases, a decrease in the percentage of patently killed hosts was noted. A higher percentage of grasshoppers died earlier and contained lower titres of hyphal bodies. If these observations are valid under natural conditions, late season

E. grylli epizootiology would be affected by the premature death of hosts containing lower titres of viable resting spores. In grasshopper management programs, E. grylli Pathotype 2 may prove useful in producing patently killed early season grasshoppers, providing inoculum that may augment other naturally occurring agents in suppressing late season host populations.

Clearly, the research presented herein only constitutes a starting point for further investigation. The interrelationships between climate, host, and pathogen require more extensive investigation to determine the utilization of E. grylli against grasshoppers. Epizootiology among early season immature grasshoppers providing increased inoculum for dramatic mid-season epiootics among adults represents an important area of research not addressed in this investigation. The development of artificial media capable of supporting the culture of E. grylli would greatly enhance the likelihood for utilization of this pathogen in grasshopper population management.

CONCLUSION

The information gained in this investigation has provided a starting point for further research into the culture, purification, and epizootiology of E. grylli Pathotype 2.

Artificial media capable of supporting the complete life cycle of E. grylli is unknown and represents a major obstacle in the study of this organism. The method of production and density-gradient purification of germ conidia employed in this study was significant in that discreet, experimental batches of germ conidia were obtained that could be formulated and applied with greater flexibility than with conventional canopy-type conidium isolation. Injections of germ conidia suspended in saline/glycerol (30v/70v) proved to be an easy, reliable method of infecting Melanoplus grasshoppers providing for the subsequent production of fresh resting spores.

Investigation into the agent(s) and mode(s) of infections with Pathotype 2 has indicated that cuticular invasion by germ conidia is the means that Melanoplus grasshoppers become infected. Scanning electron micrographs revealed no germination of germ conidia on the visible host integument at 90% relative humidity. Host mortality from E. grylli significantly increases as the macro-humidity levels approach 95% relative humidity. Significantly higher mortality was obtained with grasshoppers incubated in constant light suggesting a negative phototropic response in germ conidia germ tubes. Additional investigation on phototropism in all life stages of entomopathogenic fungi would be beneficial. Ingestion of any life stage is not a

factor in disease transmission, although dissemination of viable resting spores in fecal material may occur. Utilization of individual cages for holding grasshoppers during bioassays aided in controlling exposure to secondary sources of inoculum, cannibalism, and fortuitous pathogens, providing for more accurate interpretation of treatment results.

Fig. 1. Individual grasshopper bioassay cages.

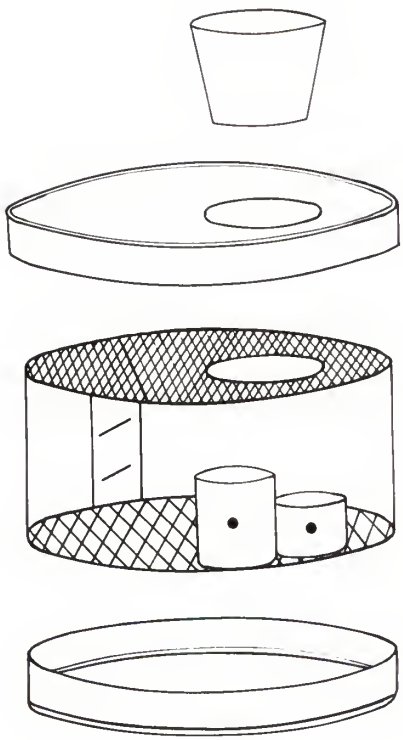
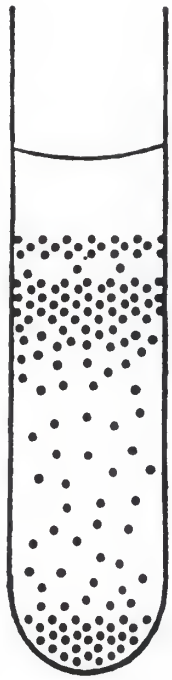


Fig. 2. Percoll density-gradient profile showing buoyant densities of germinated resting spores, germ conidia, and non-germinated resting spores.

Density Gradient Profile



	Density (g/ml)
GRS	1.040-1.050
GC	1.055-1.085
NGRS	1.080-1.120

Starting density = 1.070 g/ml

Fig. 3. Separation of E. grylli Pathotype 2 banded life stages as influenced by Percoll gradient starting density.

Band Separation vs. Gradient Starting Density

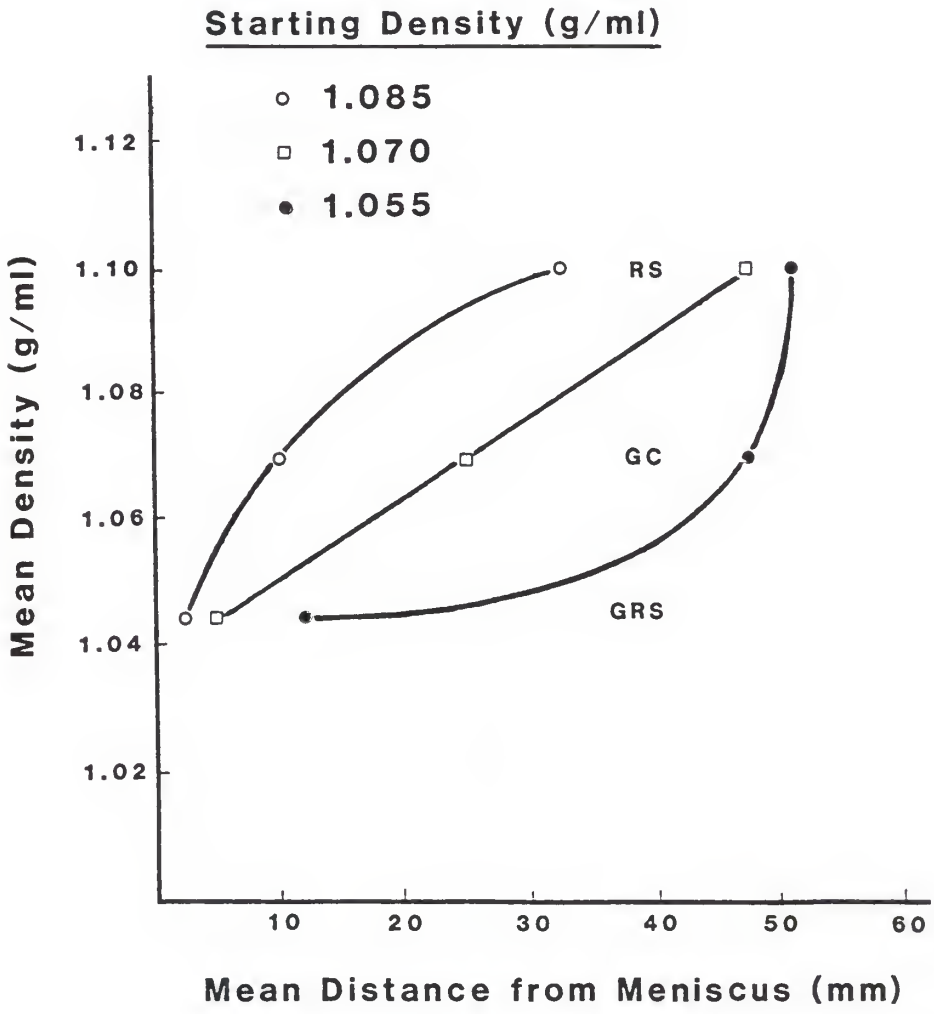


Fig. 4. Cummulative percent mortality of adult M. differentialis following injection of an average of 744 E. grylli Pathotype 2 germ conidia.

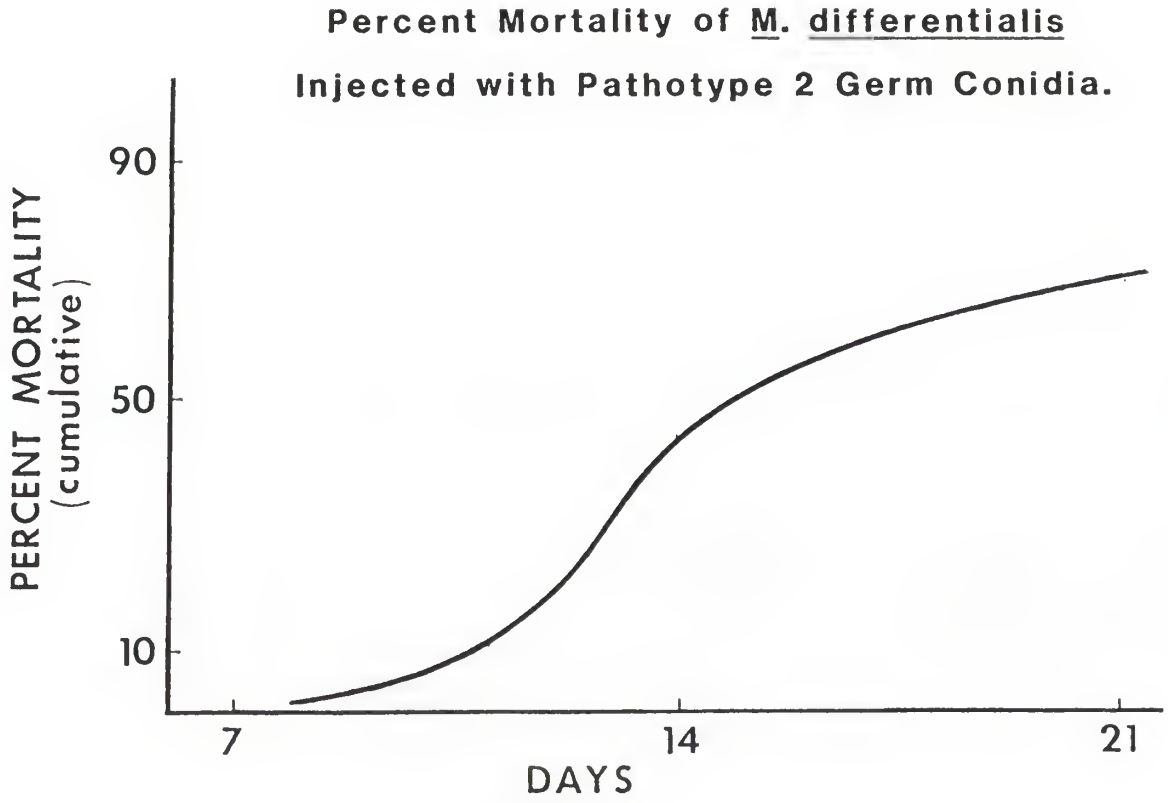

Percent Mortality of M. differentialis
Injected with Pathotype 2 Germ Conidia.
PERCENT MORTALITY
(cumulative)
90
50
10
7
14
21
DAYS

Table 1. Comparison of injection and *per os* pathogenicity of *E. grylli* Pathotype 2 life stages against adult *M. differentialis*.

Entomophaga grylli, Pathotype 2
Injection and *Per os* Bioassay

Treatment[a]		Dose/Dosage/ml	% Mortality	
			E. grylli	Other
HB	I	6.69×10^6	71.4	28.6
	P		0	14.3
GC	I	6.20×10^5	100	0
	P		0	14.3
GRS	I	3.00×10^5	85.7	14.3
	P		0	0
RS	I	1.38×10^6	100	0
	P		0	0
Controls				
S+G[a]	I	—	0	28.6
	P	—	0	0
NT		—	0	0

a/ 30:70 Saline : Glycerol suspension

HB, hyphal bodies; GC, germ conidia;

GRS, germ. resting spores; RS, resting spores;

I, injection; P, *per os*

Table 2. Dermal infectivity of *E. grylli* Pathotype 2 life stages against *M. differentialis* (95% RH, 24°C, 48-hr. post-treatment incubation).

Entomophaga grylli, Pathotype 2 Topical Bioassay

Treatment[a]	Dosage	N	% Mortality	
			E. grylli	Other
GRS	180/cm^2	28	3.5	28.6
GC	812/cm^2	28	32.1	21.4
RS	1835/cm^2	28	0	25.0
Control	——	14	0	21.4

a/ GRS, Germinated Resting Spores

GC, Germ Conidia

RS, Resting Spores

Table 3. Dermal infectivity of *E. grylli* Pathotype 2 germ conidia as influenced by relative humidity and light.

Mortality of M. differentialis from Spray Application of E. grylli, Pathotype 2 Germ Conidia

		Percent Mortality				
	Viability	100% RH		90% RH		
Rep	($x10^5$)	Light[a]	Dark	Light	Dark	C
1	2.70 (18.3)[b]	15	0	5	5	0
2	1.32 (16.5)	35	10	—	—	0
3	3.16 (30.1)	35	20	20	20	0
Mean	2.39 (21.6)	28.3	10	12.5	12.5	0
SE	———	6.67	4.08	7.50	7.50	0

a/ Significant difference between 100% light and dark treatments by pooled Binomial Test ($0.01<p<0.02$)

b/ Percent germination of germ conidia

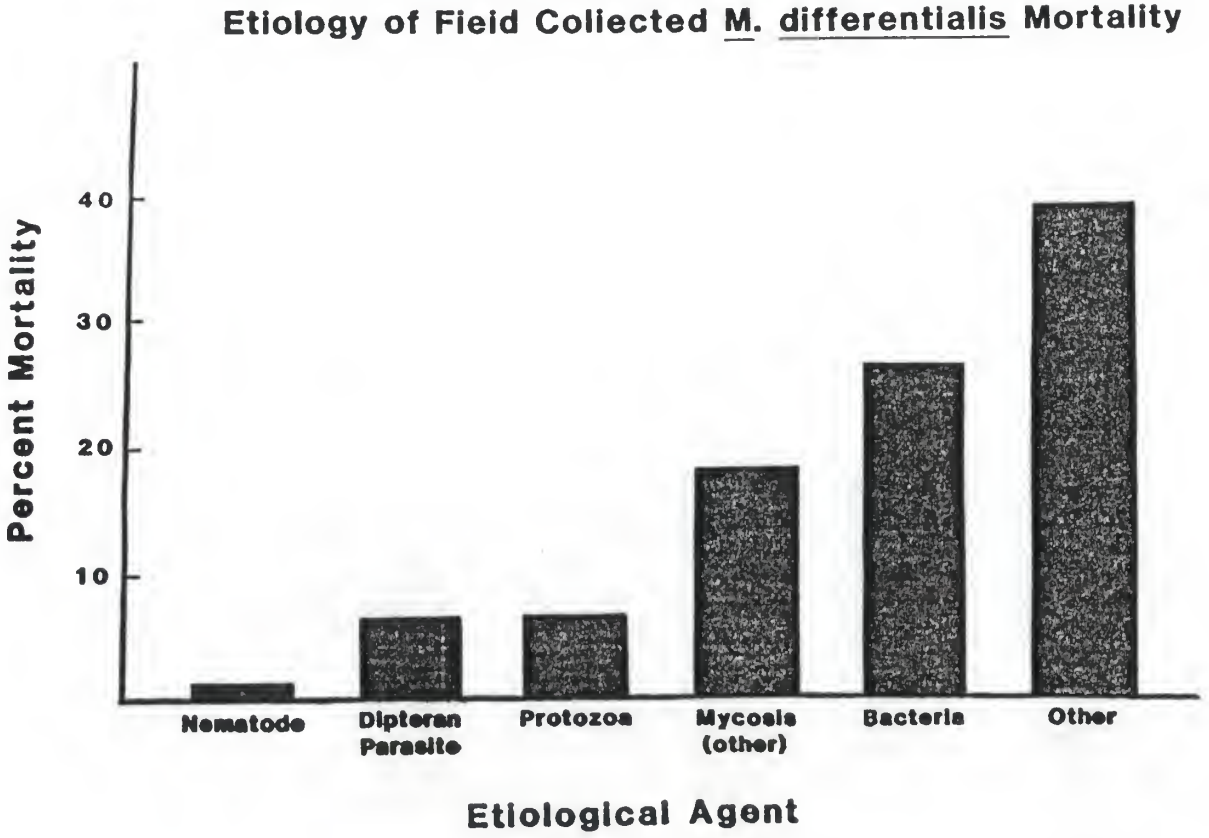
Fig. 5. Germ conidia germination on the host integument following 8 hours incubation at 90% RH.

Fig. 6. Germ conidia germination on the host integument following 8 hours incubation at 100% RH.

Fig. 5. 90% RH

Fig. 6. 100% RH

Fig. 7. Percent mortality of older, field collected M. differentialis attributed to various etiological agents.


Etiology of Fieid Coliected M. differentialis Mortality
Percent Mortality
40
30
20
10
Nematode
Dipteran Parasite
Protozoa
Mycosis (other)
Bacteria
Other
Etiological Agent

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PURIFICATION AND INFECTIVITY OF *ENTOMOPHAGA GRYLLI* (FRESENIUS) BATKO, PATHOTYPE 2 AGAINST *MELANOPLUS DIFFERENTIALIS* (THOMAS)

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AN ABSTRACT OF A MASTER'S THESIS

Submitted in Partial Fulfillment of the Requirements
for the Degree Master of Science

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1984

ABSTRACT

Resting spores of Entomophaga grylli (Fres.)Batko, Pathotype 2, were germinated in sterile, distilled water in 25 cm^2 tissue culture flasks. Following transfer onto 1% water agar, germ conidia were produced from germinated resting spores by air-drying in a laminar flow hood. Purification of the fungal life stages was accomplished by centrifugation in Percoll^{TM} density-gradient medium. The range of buoyant densities for germinated resting spores, germ conidia, and resting spores respectively was: 1.040-1.050, 1.055-1.085, and 1.080-1.120 g/ml. Experimental batches of germ conidia with greater than 98% purity were obtained with density-gradient centrifugation.

Per os bioassay of all stages in the life cycle of Pathotype 2 against adult Melanoplus differentialis (Thomas) yielded no infections. Viable resting spores were recovered from host fecal material.

Topical bioassay of resting spores, germinated resting spores, and germ conidia indicate the germ conidium is the infective agent. Significantly higher mortality among M. differentialis was seen with hosts incubated at 100% RH compared with hosts incubated at 90% RH for 24 hr. following spray application of germ conidia. Significantly higher mortality was also obtained with hosts incubated in the light at 100% RH suggesting a negative phototropic response in Pathotype 2 germ conidia.