ISOLATION AND CULTURE OF
ENTOMOPHTHORA GAMMAE,
A FUNGAL PARASITE OF NOCTUID LARVAE

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ABSTRACT

Entomophthora gammae (Weiser) was isolated by allowing conidia from infected soybean looper, Pseudoplusia includens (Walker), larvae to shower onto egg yolk agar. Fungal and bacterial contaminants were always present, but at 20°C, E. gammae grew fast enough to allow the subculture of hyphal bodies to clean medium. Once isolated, the fungus was cultured on Czapek solution agar, potato dextrose agar, Sabouraud dextrose agar, and Sabouraud maltose agar, all containing 0.2% yeast extract, and on Grace's insect tissue culture medium. It did not grow on Wolf's medium, potato agar or potato dextrose agar without yeast extract. The fungus grew most rapidly in culture when maintained at 25°C and 100% RH. Conidia on glass slides held at 25°C germinated only at 100% RH. A gradual increase in conidium size occurred over a 10-month period of continuous culture. Attempts to infect larvae with the cultured fungus were unsuccessful.

RESUMEN

Entomophthora gammae (Weiser) fue aislado permitiendo que las conidias provenientes de las larvas del medidor de la soya, Pseudoplusia includens (Walker) se dispersaran sobre agar de la yema de huevo de gallina. Hongos y bacterias contaminantes estuvieron siempre presentes, pero a 20°C, E. gammae creció lo suficientemente rápido para permitir la subcultura de cuerpos hifales para clarificar el medio. Una vez aislado, el hongo fue cultivado en una solución de agar Czapek, papa dextrosa agar, Sabouraud dextrosa agar y Sabouraud maltosa agar, todos los medios contenían 0.2% de extracto de levadura y sobre el medio Grace para cultivo de tejidos de insectos. No hubieron crecimientos sobre el medio Wolf, papa agar o papa dextrosa agar cuando no se agregó extracto de levadura. El crecimiento máximo y la germinación de las esporas ocurrió a los 25°C y con un 100% de humedad relativa. Un incremento gradual del tamaño de las conidias ocurrió durante los 10 meses de cultivos continuos. Intentos para infestar larvas con el hongo cultivado no tuvieron éxito.

Several species of the noctuid subfamily Plusiinae are susceptible to infection by Entomophthora (Tarichium) gammae (Weiser). Harper and Carner (1979) reported this pathogen from soybean looper, Pseudoplusia includens (Walker), and cabbage looper, Trichoplusia ni (Hübner), and Weiser (1965) reported it from Plusia gamma (L.) in Europe. It is a widespread pathogen of P. includens in the southeastern United States where this pest attacks soybean (Harper et al. 1983). Several studies on this pathogen have defined environmental conditions which are related to its ability to induce and maintain epizootics (Newman and Carner 1974, 1975a,b). This report presents results of work on the isolation and culture
of *E. gammae*, on defining conditions for *in vitro* growth of the fungus, and on laboratory infectivity studies.

**METHODS AND MATERIALS**

**ISOLATION**

Fourth- and fifth-instar *P. includens* larvae were collected on soybeans in fields near Tallahassee, Alabama. At the time of collection, an epizootic of *E. gammae* was in progress. Larvae were placed in individual 30-ml cups containing meridic diet (Shorey and Hale 1965) and returned to the laboratory. Larvae infected with *E. gammae* typically died in late afternoon and rapidly turned an abnormal pale yellow color. Dead larvae were taped to the inside of the lids of 20 × 100 mm petri dishes containing either egg yolk agar (EYA), potato agar, potato dextrose agar (PDA), Sabouraud maltose agar, Sabouraud dextrose agar, or Wolf's medium (Wolf 1951). Plates of each medium were held at 20 and 25°C. By the following morning, conidiophores and conidia had been produced, the latter having showered onto the medium surface below.

Once *Entomophthora* could be identified microscopically on the plates, it was transferred to fresh medium using a sterile inoculating needle. By transferring at this stage, contaminants, which were always numerous, were left behind. Several passages were sometimes necessary to completely eliminate all contaminants.

**CULTURE**

Once isolated, small quantities of hyphae were transferred with sterile inoculating loops to the centers of plates containing various media or into tubes of liquid media and maintained at 20 or 25°C. Transfers were made every 14 days to maintain actively growing cultures. Cultures were also held at 10°C and transferred every 2 months without loss of viability.

**GROWTH AND GERMINATION**

Plates of EYA and PDA plus 0.2% yeast extract were inoculated with *E. gammae* as described above and incubated in temperature cabinets maintained at 5 ± 1 degree intervals from 5 to 35°C. Colony size was determined daily for 13 days by placing each dish over a cool light source and recording the average of two diameter measurements taken at right angles to each other.

Seven petri dishes containing pure sporulating cultures of *E. gammae* were inverted over seven EYA plates and allowed to shower for 3-4 hours. Inoculated plates were covered and maintained at the temperatures used in the growth rate studies. Plates were examined daily under a microscope to determine the percent germination of 100 conidia.

Humidity chambers were established using glass desiccator jars containing saturated salt solutions to provide the following humidity levels (Solomon 1951): <1% (CaCl₂ without water), 30% (CaCl₂ · 6H₂O), 50% [CO(NO₃)₂ · 4H₂O], 70% (NH₄Cl + KNO₃), 90% (ZnSO₄ · 7H₂O), 100% (distilled water). Humidity in each chamber was checked with a humidity
gauge (Bacharach Industrial Instrument Co.), allowing sufficient time after
closure for the humidity level to stabilize.

Sporulating cultures of *E. gammae* were inverted over glass slides for
3-4 h, and the slides were introduced into the chambers. All chambers were
held at room temperature (ca. 25°C) under fluorescent lighting. Slides were
removed from the chambers at ca. 5-h intervals for 1 day and again at 57 h.
Percent germination was determined at each interval from the first 100
conidia encountered while scanning the central area of the slide.

**INFECTIVITY STUDIES**

Field collected cadavers exhibiting symptoms of *E. gammae* infection
were suspended over individual healthy, laboratory-reared *P. includens*
larvae held inside petri dishes. Seven larvae were successfully exposed in
this manner. Ten larvae were exposed to conidia showering from each of 3
separate, inverted culture plates which had been maintained in the labora-
tory through 17 transfers over a 1-year period. After 4-6 hours exposure, all
larvae were transferred to individual 1-ounce cups containing diet and were
held at either 90 or 100% RH until they pupated or died.

**RESULTS**

**ISOLATION**

Although several media supported growth of *E. gammae*, EYA held at
20°C was the only medium-temperature combination from which a pure
culture could be initiated. The inoculation technique introduced many bac-
terial and fungal contaminants which at 25°C overgrew the plates before
*E. gammae* colonies could become established. These contaminants grew
more slowly at 20°C on EYA, and *E. gammae* colonies were able to develop
vegetatively and could be transferred to clean plates before being over-
grown. After the transfer of hyphal bodies, the mycelium grew vegetatively
for 2-3 days; then hyphal bodies produced conidia which were ejected.
Those landing on fresh medium germinated, produced germ tubes which
elongated, thickened and grew vegetatively, repeating the above cycle. This
process continued as a series of expanding concentric rings of growth until
the petri dish limited further growth.

**CULTURE**

Hyphal bodies transferred to media other than EYA exhibited varying
growth responses. Potato agar, Wolf's medium, and PDA did not support
growth. A very weak growth was obtained in Czapek solution containing
0.2% yeast extract. PDA + 0.2% yeast extract supported good growth,
while Sabouraud dextrose agar and Sabouraud maltose agar, both contain-
ing 0.2% yeast extract, supported even greater growth. Greatest growth
occurred on EYA containing 0.2% yeast extract and on Grace's insect tissue
culture medium. On EYA held at 25°C, *E. gammae*, following the growth
cycle described above, covered a 10 cm plate within 1 week. On Grace's
medium, the inoculum floated to the surface and grew as a white, compact
mass.
GROWTH AND GERMINATION

Growth on EYA was so rapid and diffuse that growth rates were difficult to determine. PDA + yeast extract supported slower growth with more clearly defined colony borders; growth on this medium at 5 to 35°C is presented in Table 1. Growth was clearly inhibited at the two temperature extremes. Most rapid growth occurred at 25°C, followed in order by 20 and 30°C. Growth was negligible at 10 and 15°C. Conidial germination and hyphal development occurred equally well at 20, 25, and 30°C, but no germination or growth was observed at 5, 10 or 35°C (Table 2).

In humidity chambers, 20% of conidia on glass slides germinated after 6 hours at 100% RH and one conidium germinated after 12 hours at 90%; no germination occurred through 57 hours at any of the other RH levels tested.

Distinct morphological changes occurred in E. gammae cultured continuously on EYA for 10 months. Naturally occurring conidia and those produced by initial isolations were the typical shape and size (18.80 ± 2.20 x 8.80 ± 1.60 μm) reported for the fungus (Harper and Carner 1973). After 10 months in culture, conidia were more oval, larger in size (28.77 ±

### TABLE 1. GROWTH OF Entomophthora gammae AT DIFFERENT TEMPERATURES ON POTATO DEXTROSE AGAR CONTAINING YEAST EXTRACT.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Colony diameter (mm), at day after inoculation (n = 3 plates)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10  11  12  13</td>
</tr>
<tr>
<td>10</td>
<td>0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.5 0.8 0.8 1.2</td>
</tr>
<tr>
<td>15</td>
<td>0.3 0.3 0.3 0.3 0.3 0.3 0.3 0.6 1.2 1.4 1.9 2.9 3.1 4.6</td>
</tr>
<tr>
<td>20</td>
<td>0.3 0.3 1.1 2.3 4.2 5.8 7.3 9.8 12.8 19.8 23.8 28.8 33.8</td>
</tr>
<tr>
<td>25</td>
<td>0.3 1.1 2.1 5.6 7.1 10.6 13.6 19.6 24.6 31.6 36.6 38.6 43.6</td>
</tr>
<tr>
<td>30</td>
<td>0.3 0.3 0.4 2.1 3.1 4.9 6.6 8.6 11.6 14.6 16.6 20.6 23.6</td>
</tr>
</tbody>
</table>

¹No growth occurred at 5 or 35°C. All plates were inoculated with a 0.3 mm diameter mass of hyphae.

### TABLE 2. CONIDIAL GERMINATION AND GROWTH OF Entomophthora gammae ON EGG YOLK AGAR AT DIFFERENT TEMPERATURES FOLLOWING INOCULATIONS

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>% Germination¹</th>
<th>Growth²</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h 72 h</td>
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<tr>
<td>10</td>
<td>0</td>
<td>0</td>
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<tr>
<td>15</td>
<td>0</td>
<td>0</td>
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<td>60</td>
<td>95</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

¹Based on examination of 100 conidia at each temperature.
²0 = No growth, + = poor growth, ++ = Good growth, +++ = Very good growth.
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4.34 x 19.85 ± 4.65 μm), and contained larger and more numerous oil globules. In culture, conidiophores were difficult to find. Conidia were normally formed and ejected from the tips of the stocky hyphal bodies. The membrane or gelatinous layer which surrounds the naturally occurring conidium is often quite thick in relation to conidium size (Harper and Carner 1973). After continuous culture, this layer became very thin.

Repeated attempts to infect P. includens larvae with conidia from cultures of E. gammae were unsuccessful, and only one of 7 larvae became infected after exposure to freshly sporulating, field collected larvae. The infected larva died after 7 days, became mummified, retained its shape, and became glossey black in color. On dissection, the cadaver was found to be filled with typical E. gammae resting spores as described by Harper and Carner (1973).

DISCUSSION

There is considerable information on optimal conditions for growth and development of many species in the Entomophthoraceae. Members of the Entomophthoraceae vary considerably in their nutritive requirements. Coagulated egg yolk or media containing egg yolk has proven satisfactory for culturing many species (see review by King and Humber, 1981). EYA was clearly the optimal solid growth medium for E. gammae in this study. Once isolated, the fungus grew on a number of standard mycological media if supplemented with yeast extract. While resting spores are commonly produced by E. gammae under field conditions (Harper and Carner 1973, Newman and Carner 1975b), they were never produced on any medium under any of the conditions utilized in this study.

Temperature requirements for growth of the Entomophthoraceae vary considerably (Gustafsson 1965, Hall and Bell, 1960, 1961). In this study, the optimal temperature for growth of E. gammae in vitro was 25°C or slightly lower. Spore germination on medium also appeared to be optimal in the same temperature range, which agrees with an earlier report by Newman and Carner (1975a), who found that sporulation by E. gammae on cadavers was maximal at 21 and 26.7°C.

The procedures used to determine optimal humidity conditions for spore germination served only as an indication of this requirement, as the response was very low. However, it is clear that germination occurred only at or near conditions of 100% humidity, which is also in agreement with results of Newman and Carner (1975a). They found no germination of conidia of this species at 90% RH, only 1% at 95% RH, and 95% at 100% RH.

Lack of infectivity by the cultured fungus may have resulted from the environmental conditions or techniques employed, or from a loss of infectiousness following repeated subculturing. The low level of infectivity using fresh cadavers as the inoculum source suggests that the inoculation technique was inadequate. However, the gradual change in spore morphology with continued culture indicates that a selection process was occurring, possibly for an organism that was better adapted for saprophytic growth at the expense of pathogenicity. This phenomenon would reinforce findings with other pathogens which have shown the importance of maintaining minimal passage stock cultures to insure maintenance of desirable char-
REFERENCES CITED


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**LIRIOMYZA TRIFOLII**:

**OVIPosition AND DEVELOPMENT IN FOLIAGE OF TOMATO AND COMMON WEED HOSTS**

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**ABSTRACT**

Oviposition and development of *Liriomyza trifolii* (Burgess) were studied in the laboratory in foliage of tomato, *Lycopersicon esculentum* Mill. cv. 'Walter,' a common nightshade, *Solanum nodiflorum* Jacq., common beggar-tick, *Bidens alba* (L.) D.C., and downy groundcherry, *Physalis pubescens* L. *Liriomyza trifolii* oviposited successfully in foliage of all plant