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Characterization of the Fungal Pathogen, *Entomophaga aulicae* (Zygomycetes: Entomophthorales) in Larval Populations of the Green Cloverworm, *Plathypena scabra* (Lepidoptera: Noctuidae)

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Abstract: *Entomophaga aulicae* was monitored in populations of noctuid lepidopteran pests of soybean during the 2000, 2001, and 2002 growing seasons in South Carolina and infected only *Plathypena scabra* larvae. No infection by *E. aulicae* was detected in 2002. Average infection levels of *E. aulicae* in *P. scabra* populations in Blackville were 6.0% and 20.0% in 2000 and 2001, respectively. At Clemson, infection was 15.0% and 23.3% for the two sampling weeks in 2000, and infection reached a high of 50.0% in 2001. Pyriform, multinucleate conidia and spherical to slightly oval resting spores were the primary features of this fungus. When conidia were kept in a high humidity for 6-12 hrs they either formed long germ tubes or secondary conidia. Resting spores were formed by budding from parental cells. This study is the first detailed characterization of *E. aulicae* from *P. scabra*.

Key Words: Entomopathogenic fungi, Lepidoptera, Soybean.

Plathypena scabra'nın (Lepidoptera: Noctuidae) Larval Populasyonunda Fungus Pathojeni Olan *Entomophaga aulicae*'nın (Zygomycetes: Entomophthorales) Tanımlanması

Özet: Noctuid Lepidoptera populasyonu üzerindeki doğal pathojen *Entomophaga aulicae*'nın 2000, 2001, ve 2002 tarım sezonunda oluşumu araştırıldı ve sadece *P. scabra* larvasında infeksiyon gözlendi. 2002'de *E. aulicae* tarafından infekte edilmiş noctuid larvasına rastlanmadı. Blackville'deki *P. scabra* populasyonunun ortalama infeksiyon düzeyi 2000 yılında % 6 ve 2001 yılında % 20 düzeyinde gözlenmiştir. Clemson'da infeksiyon 2000 yılında %15 ve % 23 ve 2001 yılında infeksiyon oranı % 50'ye kadar ulastığı gözlendi. *Entomophaga aulicae*'nın başlıca özellikleri pyriform şeklinde ve çok çekirdekli conidia, ve küresel'den ovalle kadar uzanan dinlenme sporlarına sahip olmasıdır. Conidialar yüksek neme 6-12 saat maruz bırakıldıkları zaman uzun tohum tüpleri yada ikincil conidia oluştururlar. Dinlenme sporları ana hücrelerden tomurcuklanma ile oluşurlar. Bu çalışma *E. aulicae*'nın *P. scabra*'da karakterize edildiği ilk çalışmadır.

Anahtar Sözcükler: Fungus pathojenleri, Lepidoptera, Soya fasülyesi.

Introduction

Entomophaga aulicae (Reichardt in Bail) Batko is an important fungal pathogen of many species of Lepidoptera. This species was first reported under the name *Empusa aulicae* by Reichardt as reported in Bail (1) and later transferred to the genus *Entomophthora* (2). Finally, Humber (3) placed this fungus in the genus *Entomophaga*. *Entomophaga aulicae* is considered as a complex of species. Species complex whose components cannot be differentiated readly on a basis of their morphologies. Restriction length polymorphism (RFLP), allozyme techniques and host specificity have been used to distinguish members within this complex (4-6). The only two species currently formally recognized in this complex are *Entomophaga maimaiga* Humber, Soper and Shimazu and *E. aulicae*.

Entomophaga aulicae has been reported from major forest defoliators such as the eastern hemlock looper, *Lambdina fiscellaria* (Guenée) (7), the eastern spruce budworm, *Choristoneura fumiferana* (Clemens), (8), the

brown tail moth, *Euproctis chrysorrhoea* (Linnaeus), (9) and the salt-marsh caterpillar, *Estigmene acrea* (Drury) (10).

Several *Entomophthorales* have been reported infecting species of noctuids in soybean in South Carolina (11, 12). *Pandora gammae* (Weiser) Humber [as *Entomophthora gammae*] was reported from the loopers, *Pseudoplusia includens* (Walker), and *Trichoplusia ni* (Hübner) and an unidentified species of *Entomophaga* [as *Entomophthora*] from *P. scabra*, and the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner. Hamm (13) found a similar species infecting the corn earworm, *Helicoverpa zea* (Boddie), the sorghum webworm, *Celama sorghiella* (Riley), the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) and the tobacco budworm, *Heliothis virescens* (Fabricius), and identified it as *Entomophthora aulicae*.

Plathypena scabra (F.) is found in almost every soybean field in the United States at some time during each growing season. It is usually present in South Carolina soybeans early in the season and seldom reaches pest status. Beneficial insects and pathogens usually regulate *P. scabra* populations below economic injury levels in most areas. Low densities of *P. scabra* often are beneficial in harboring pathogens so that high levels of inoculum can be maintained for infection of later pest populations (14).

During evaluation of the role of *Nomuraea rileyi* in soybean fields in South Carolina, another fungal pathogen, *E. aulicae* was observed infecting the green cloverworm, *P. scabra*. During the 2000 and 2001 growing seasons, this fungus caused higher mortality in *P. scabra* than *N. rileyi*. The objectives of this study were to identify and to characterize *E. aulicae* from *P. scabra*, to monitor the incidence of *E. aulicae* in noctuid larvae in soybean fields in South Carolina, and to describe the developmental stages of the fungus.

Materials and Methods

Larvae of *P. scabra*, *A. gemmatalis, P. includens, H. zea, S. exigua* and fall armyworm, *Spodoptera frugiperda* (Hübner), were sampled weekly from 31 August-10 October 2000, 31 July-9 October 2001 and 7 July-2 October 2002 at the Edisto Research and Education Center, Blackville, South Carolina. Samples from the Simpson Farm, Clemson SC were taken on September 25 and October 5, 2000 and from July 16 through October 4, 2001. In 2002, soybean fields at Clemson were treated with insecticide, and no noctuid larvae were collected. Larvae were collected from untreated soybean fields by taking 10-15 one-meter ground cloth samples from each field (15) that were placed on artificial diet and kept at 27 ± 1 °C, 75% RH with a photoperiod of 16:8 h (L:D). Collected larvae were checked daily for infection by *Entomophaga* species.

Fresh cadavers of *E. aulicae*-infected larvae were placed on glass slides for several hours in moist Petri dishes to collect conidia. Resting spores were collected by removing portions of infected tissue. Conidia and resting spores were mounted in lactophenol-cotton-blue and in lactophenol-aceto-orcein to stain nuclei for light microscopy examination. Fungal structures from infected larvae were fixed in 2% osmium tetroxide vapors for 48 h. Osmium-fixed samples were directly coated with gold (Denton Vacuum, LLC Desk II Cold Sputter Unit) and coated samples were examined with a Hitachi S-3500N scanning electron microscope at 20 kV accelerating voltage. Measurements of fungal structures were collected from digital images using a Quartz PCI-Image Management System.

Results

Symptoms of *E. aulicae* infection

Healthy *P. scabra* larvae were pale green with often two white stripes on each side of the body. *Entomophaga aulicae* infected larvae in the field were usually fixed to upper parts of the plants by their hind legs. Cadavers turned yellow-green or light tan. At death, larvae which contained the resting spore stage could not be distinguished from those with the conidial stage. However, if larvae were held at high humidity for several hours, conidiophores appeared on the surface of conidial stage larvae. No external fungal growth developed on larvae containing the resting spore stage (Figure 1). Conidial production began ~4 hr after the larvae died and continued for about 12 hr. During conidial production, larvae began to shrink and became wrinkled and misshapen (Figure 2).

Description of *E. aulicae*

Conidia were formed directly on the apex of simple conidiophores without a neck or constriction. The

primary conidia were pyriform to obovate with a uniformly oval apex, and broad, rounded papilla at the base (Figure 3). Conidia were forcibly discharged by papilla eversion. Aceto-orcein stain revealed 4-8 granular nuclei per conidium. Measurements for the primary conidia were 28.0 ± 3.0 µm x 19.5 ± 1.9 µm (n = 50, range = 22.8 - 35.0 x 17.5 - 23.3).

When conidia were kept at 100% RH for 6-12 hrs they either formed long germ tubes or developed a short stalk on which a secondary conidium was formed. Contents of the primary conidium moved to the secondary conidium and left behind the cast of the primary conidium. Secondary conidia were similar in shape, and smaller than, primary conidia (*t*-tests; $P < 0.05$, df = 49). Secondary conidia dimensions were 18.5 ± 1.5 µm x 14.1 ± 2.0 µm (n = 50, range = 17.5 - 22.7 x 10.0 - 17.5). Conidia were forcibly discharged and adhered to the walls of the diet cup.

Dead larvae which did not show signs of external fungal growth after several hours at high humidity usually contained the resting spore stage of *Entomophaga*. These larvae were filled completely with spherical to slightly oval spores. Resting spores were formed by budding from parental cells. The walls of these resting spores were quite thin compared to most resting spores of other species of *Entomophthorales*. Their dimensions were 34.5 ± 4.5 µm x 34.4 ± 4.5 µm (n = 20, range = 19 - 40.0 x 19.5 - 40.2) (Figure 4).

If the contents of larvae infected with the resting spore stage were examined shortly after the larvae died, various stages in the development of the spores could be seen. Resting spores apparently developed as outgrowths of single irregular-shaped hyphal bodies, so they are probably azygospores. The spores appear to begin as a small evagination on one side of the hyphal bodies, and as they enlarge, the contents of the hyphal bodies move into

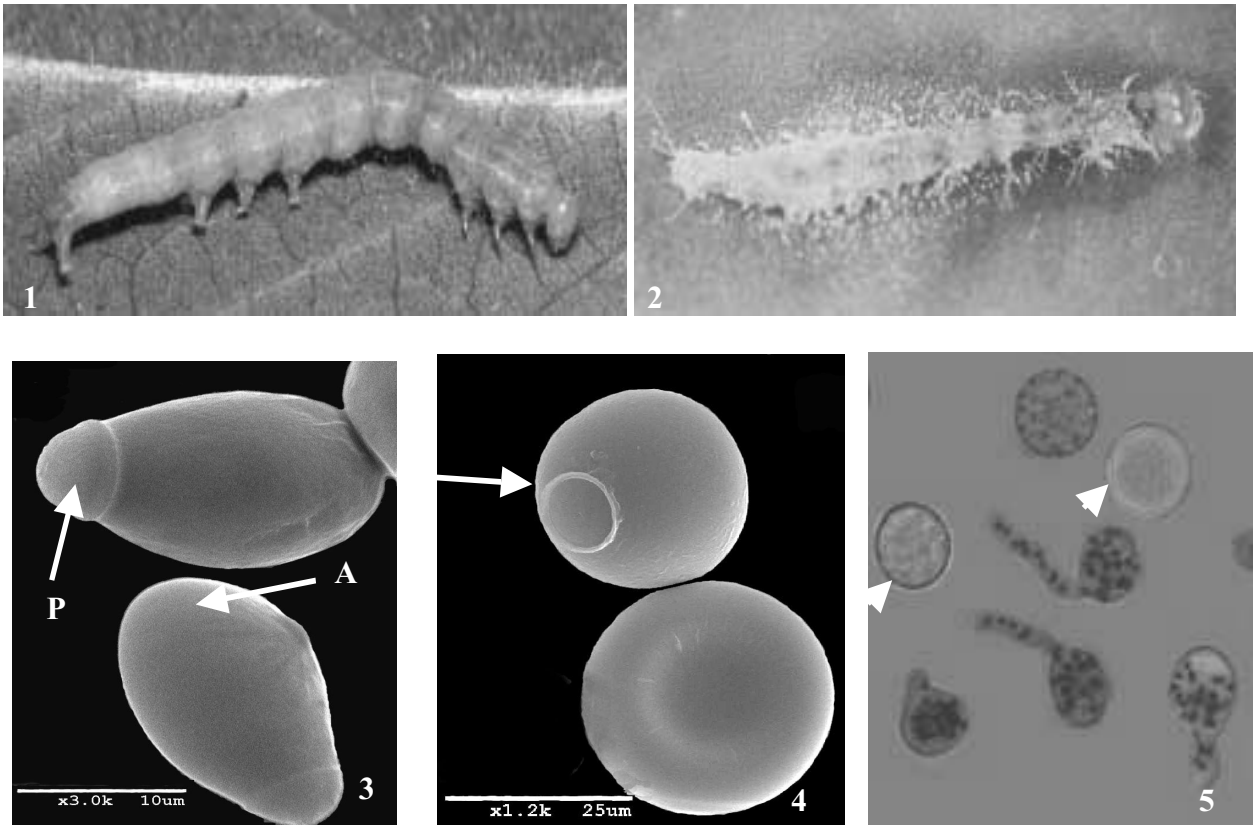

Figure 1. *Plathypena scabra* larva infected with the resting spore stage and (2) with the conidial stage of *Entomophaga aulicae* showing discharged conidia. (3) Scanning electron micrograph showing pyriform to obovate primary conidia with oval apex (A), and flat to rounded papilla (P) Bar = 10 µm, (20 kV, x3.0k) and (4) spherical to slightly oval resting spores of *Entomophaga aulicae*. Ring shows location of previous hyphal body attachment, Bar = 25 µm, (20 kV, x1.2k). (5) Resting spore development of *Entomophaga aulicae* from single irregular shaped hyphae bodies. Arrows indicate mature resting spores.

the developing spores. After the spores complete development, the empty hyphal bodies drop off. At the beginning of resting spore formation, nuclei were stainable with aceto-orcein; however, no stained nuclei were seen in mature spores because of the stain's inability to penetrate the spore wall. Twenty to 40 nuclei were counted in the developing resting spores (Figure 5).

Larvae infected with the conidial stage of *E. aulicae* also contained a few resting spores inside the infected host tissue. These spores had thicker walls and were somewhat smaller than the resting spore described above. These thick-walled spores were more typical for resting spores described for other species of *Entomophaga*.

Disease Prevalence

Anticarsia gemmatalis, *P. scabra*, and *P. includens* were observed in soybean fields but only *P. scabra* was infected with *E. aulicae* in two sampling seasons, 2000 and 2001.

Entomophaga aulicae was observed in *P. scabra* populations from September 20 through October 04 in 2000 (Table 1). The two samples taken at Clemson yielded 15% and 23.3% infection with *E. aulicae* on September 25 and October 05 2000, respectively (Table 2).

In Blackville in 2001, infection by *E. aulicae* over the entire season was about the same, with 13% of total *P. scabra* larvae collected. *Entomophaga aulicae* was observed from mid-August through early October, reaching a peak of 30% infection on September 25 (Table 3). At Clemson, infection was observed one week earlier than in Blackville and reached as high as 50% on September 15 (Table 4). In spite of having relatively high infection by *E. aulicae* in 2000 and 2001, this fungus was not observed at all during the 2002 season. Infection of *N. rileyi* was also very low (1.7% of total *P. scabra* larvae) (16 unpublished data).

Table 1. Mortality of green cloverworm (GCW) larvae by *Entomophaga aulicae* collected from soybeans at Blackville, SC, 2000.

Date	Total Larvae Collected [a]	% GCW [b]	No. of GCW	% Mortality Caused by *Entomophaga*
08/31/00	169	67	114	0.0
09/13/00	235	62	146	0.0
09/20/00	343	52.7	181	10
09/27/00	278	15	42	19
10/04/00	231	1.7	4	50
10/11/00	200	0.0	0	0.0
10/18/00	230	0.0	0	0.0

[a] Species collected include, velvetbean caterpillar, green cloverworm, soybean looper, and others.
[b] Proportion of total larvae that were green cloverworm.

Table 2. Mortality of green cloverworm (GCW) larvae by *Entomophaga aulicae* collected from soybean at Clemson, SC, 2000.

Date	Total Larvae Collected [a]	% GCW [b]	No. of GCW	% Mortality Caused by *Entomophaga*
09/25/00	80	50	40	15
10/05/00	60	20	12	23.3

[a] Species collected include, velvetbean caterpillar, green cloverworm, soybean looper, and others.
[b] Proportion of total larvae that were green cloverworm.

Table 3. Mortality of green cloverworm (GCW) larvae by *Entomophaga aulicae* collected from soybeans at Blackville, SC, 2001.

Date	Total Larvae Collected [a]	% GCW [b]	No. of GCW	% Mortality Caused by *Entomophaga*
07/31/01	52	96	50	0.0
08/09/01	54	96	52	0.0
08/15/01	75	77	58	0.0
08/21/01	176	32	56	1.8
08/29/01	289	45	131	0.0
09/07/01	231	18	42	11.9
09/11/01	601	36	218	27.5
09/18/01	605	33	197	5.6
09/25/01	466	32	149	28.9
10/02/01	386	7	28	25.0
10/09/01	274	0.7	2	0.0

[a] Species collected include, velvetbean caterpillar, green cloverworm, soybean looper, and others.
[b] Proportion of total larvae that were green cloverworm.

Table 4. Mortality of green cloverworm (GCW) larvae by *Entomophaga aulicae* collected from soybean at Clemson, SC, 2001.

Date	Total Larvae Collected [a]	% GCW [b]	No. of GCW	% Mortality Caused by *Entomophaga*
08/16/01	51	100	51	3.9
08/30/01	103	80.6	83	2.4
09/06/01	69	75.4	52	19.2
09/14/01	157	67.5	106	50.9
09/20/01	148	45.3	67	14.9
09/28/01	169	22.0	37	10.8
10/04/01	164	7.3	12	0.0

[a] Species collected include, velvetbean caterpillar, green cloverworm, soybean looper, and others.
[b] Proportion of total larvae that were green cloverworm.

Discussion

The characteristics which we describe for the *Entomophaga* species from *P. scabra* are consistent with those reported previously from many hosts (2, 13, 17, 18, 5). The sizes of primary conidia, secondary conidia and resting spores that we measured were quite variable, but generally were in the range of the *E. aulicae* species complex (2, 18). Hamm (13) also reported variable sizes of conidia from three hosts, *C. sorghiella, S. frugiperda*, and *H. zea* which averaged 31.1 x 22.9 µm, 34.9 x 24.9 µm, and 42.4 x 30.4 µm, respectively. Hajek et al. (19) reported that conidiophores produced primary conidia that varied in size, and the spores produced *in vitro* were larger than those produced *in vivo*. We also observed that conidia were formed directly on conidiophores, without a neck, and were forcibly discharged and multinucleate. The resting spores were azygospores formed by budding from hyphal bodies, and no rhizoids were formed. All of these characteristics match those that have been listed for the *E. aulicae* complex.

Entomophaga aulicae was the most abundant fungal pathogen in the field during the sampling season in 2000 and infection was observed only in *P. scabra*. In 2001, infection patterns were similar to those observed in 2000. Carner et al. (11) and Gilreath et al. (12) reported an unidentified *Entomophaga* species from *P. scabra*, *H. zea*, and *A. gemmatalis* in Blackville and the Upper Coastal Plain of South Carolina and infection rates were variable between hosts and years. No descriptions of these fungi were presented, but Gilreath et al. (12) found positive

correlations of infectivity among the noctuid hosts (13). High infection rates of *E. aulicae* were reported from Georgia in sorghum fields. The four hosts, *H. zea, C. sorghiella, S. frugiperda* and *H. virescens* had infection levels of 48-100%, 74-95%, 19-40% and 2-94 %, respectively.

It may be that strains within the *E. aulicae* species complex have some level of host specificity because same strains are not able infect hosts which are reported to be normal hosts for *E. aulicae* (20, 6). There could be different strains of the *E. aulicae* species complex operating in South Carolina on different hosts. This would explain why Carner et al. (11) and Gilreath et al. (12) reported multiple hosts for *E. aulicae* while in our studies the only host found to be infected was *P. scabra*.

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