PATHOGENESIS OF CHALKBROOD IN THE ALFALFA LEAFCUTTING BEE, *MEGACHILE ROTUNDATA*

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SUMMARY

Chalkbrood is a mycosis of larvae of certain Hymenoptera including the alfalfa leafcutting bee, *Megachile rotundata*. Basic studies were conducted to determine the course of pathogenesis in afflicted larvae. *Ascosphaera aggregata* spores germinated in the midguts and penetrated the hemocoel of fourth instar larvae within 2 days after inoculation. Invasion of epidermis, tracheae and muscles followed within 3 more days. The entire hemocoel was nearly filled with mycelium at the time of death, 3-7 days after inoculation.

INTRODUCTION

Chalkbrood is a mycosis of certain hymenopteran larvae. The disease is a serious problem for alfalfa seed growers in western North America who use populations of *Megachile rotundata* (Fabricius) for pollination (STEPHEN and UNDURRAGA, 1978). The etiology of chalkbrood in this host and the signs by which it may be recognized were reported earlier (VANDENBERG and STEPHEN, 1982, 1983). Spores of *Ascosphaera aggregata* SKOU cling to emerging adults of *M. rotundata* as they chew through spore-laden chalkbrood cadavers (VANDENBERG *et al.*, 1980). Some adults carry spores throughout their lives (STEPHEN *et al.*, 1981) and may contaminate their mates, eggs, and pollen provisions. Developing larvae are invaded by spores germinating in the gut. In this paper the course of infection from ingestion of spores until host death is described.

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FIG. 1. — Head and thorax (A) and epidermis and cuticle (B) of uninfected *Megachile rotundata* larvae.
 A) Mallory triple stain. Bar = 120 μ m. B) Periodic acid-Schiff reagent.
 Bar = 30 μ m. B = brain, E = epidermis, F = fat body.
 L = lumen of the gut, M = mandible, S = silk gland.

MATERIALS AND METHODS

Larvae were reared on an autoclaved, pollen-based diet in individual wells of polystyrene titration plates (FICHTER *et al.*, 1981). Fresh diet was inoculated with spores of *A. aggregata* at a dose of 10^5 spores per larva as described by VANDENBERG and STEPHEN (1982). Disease-free third and fourth instar larvae were transferred to the inoculated diet and allowed to feed at 30 °C. Beginning at 24 hours post-inoculation and at 12 to 24 hour intervals for 8 days, larvae were fixed for histological processing in alcoholic Bouin's fluid or buffered formalin (HUMASON, 1972). Samples were dehydrated in a graded ethanol series and cleared in toluene. Alternately, dioxane was used for both the dehydration and clearing. Paraplast® — plus (Sherwood Medical, St. Louis) was used as the embedding medium. Sections were made at 8-10 µm, affixed with Mayer's albumin to glass slides, and stained with hematoxylin and eosin, Mallory's triple stain, periodic acid-Schiff reagent or Gomori's methenamine silver nitrate (HUMASON, 1972). Sections were examined for the presence of fungal growth within the host and photographed. Other cleared and dehydrated sections were examined by scanning electron microscopy.

RESULTS AND DISCUSSION

Chalkbrood pathogenesis begins with *A. aggregata* spore germination in the midgut of *M. rotundata* larvae and subsequent invasion of the host hemocoel. Uninfected *M. rotundata* larvae are shown in Fig. 1. Some gut contents including pollen grains can be seen in Fig. 1a. Healthy epidermal and fat body tissues are visible in Fig. 1b. Germ tube formation by *A. aggregata* within the host midgut and mycelial penetration of the peritrophic lining occurred within 1-3 days after inoculation of fourth instar larvae (Fig. 2). The mechanisms by which germ tubes penetrate the peritrophic lining have not been studied but may involve extracellular enzyme production or mechanical disruption of this barrier. No spores were observed to germinate on the outer surface of the host. Within 2-4 days, fungus hyphae were detected in the host hemocoel invading the epidermis, tracheae (Fig. 3) and muscles (Fig 4 a). External signs of infection are not visible until the host is near death and nearly filled with fungus mycelium (Fig. 4b, VANDENBERG and STEPHEN, 1982). Fungus sporulation occurs beneath the host cuticle (SKOU, 1975) within 1-2 weeks after host death.

Following germination in vitro in high CO₂ concentrations (KISH, 1980; STEPHEN *et al.*, 1982), mycelial cultures isolated from germ tubes of *A. aggregata* thrive on the surface of agar exposed to air and resemble those within infected hosts (VANDENBERG and STEPHEN, 1982). However, some spores will germinate in vitro when incubated in the presence of air instead of CO₂ (VANDENBERG, 1982). Neither reduced oxygen nor CO₂ requirements were reported by SKOU (1975) when isolating this fungus. Studies on pH and oxidation-reduction potentials in the guts of uninfected *M. rotundata* larvae indicate that conditions of reduced potential and near neutral pH exist (VANDENBERG, 1982). The influence of host gut conditions on spore germination by *A. aggregata* has not been explored.

FIG. 2. — *Germ tubes of Ascospheera aggregata in this midgut of Megachile rotundata larvae, near the proventriculus (A), and the peritrophic lining (B).*

A) Hematoxylin and eosin. Bar = 30 μ m. B) Mallory triple stain. Bar = 20 μ m.

Arrows = germ tubes, H = hemocoel, L = lumen of the gut.

Pe = peritrophic lining, Po = pollen, Pr = proventriculus.

FIG. 3. — *Ascophæra aggregata* mycelia in the epidermis (A) and trachea (B) of *Megachile rotundata* larvae.

A) Periodic acid-Schiff reagent. Bar = 30 μ m. B) Mallory triple stain.

Bar = 30 μ m. Arrows = hyphae, E = epidermis, F = fat body, T = trachea.

FIG. 4. — *Ascosphaera aggregata* mycelia in muscle and epidermis (A), and in the hemocoel (B) of *Megachile rotundata* larvae.

A) Scanning electron micrograph. Bar = 10 μ m. B) Gomori methenamine-silver nitrate. Bar = 40 μ m. Arrows = hyphae, C = cuticle, F = fat body, M = muscle.

The causative agent of chalkbrood in honey bees, *Apis mellifera* L., is *Ascosphaera apis* (Maassen ex Claussen) OLIVE et SPILTOIR (BAILEY, 1967; MAURIZIO, 1934), although its route of entry into the insect is open to question (GILLIAM, 1978). MAURIZIO (1934) found the fungus in the intestines of larvae from infected colonies. BAILEY (1967) suggested that chilling of larvae resulted in increased oxygen levels in the gut and allowed renewed growth of germ tubes and mycelia of *A. apis*. Other workers claim that spores of *A. apis* may germinate within the gut or outside the host and then penetrate the cuticle (GILLIAM *et al.*, 1978; GLINSKY, 1981; ROUSSY, 1962). They applied spore suspensions to mouth-parts or dorsal cuticle and observed resulting mummy formation. Although GILLIAM *et al.* (1978) observed the fungus growing on the outer surface of larvae that later succumbed to infection, neither they, GLINSKY (1981), nor ROUSSY (1962) examined gut contents for germinating spores. It is possible that application of spores to the cuticle was followed by larval ingestion of inoculum during the course of incubation. Infection followed *A. apis* spore germination in the gut. In order to rule out this possibility, means must be devised to prevent larvae from ingesting spore inoculum.

We have shown here that spores of *A. aggregata* ingested by larvae of *M. rotundata* germinate within the host midgut. Penetration of the peritrophic membrane and ramification within various host tissues follows within 1 week. External signs of infection are not apparent until shortly before host death, by which time the fungus has nearly filled the host hemocoel. Spores on the outer surfaces of hosts were not observed to germinate although some spores incubated in vitro on a nutrient medium in the presence of air will germinate (VANDENBERG, 1982).

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RÉSUMÉ

PATHOGENÈSE DU COUVAIN PLÂTRÉ CHEZ LA MÉGACHILE.
MEGACHILE ROTUNDATA

Le couvain plâtré est une mycose qui attaque les larves de certains Hyménoptères, dont la mégachile de la luzerne, *Megachile rotundata*. On a mené des études de base afin de déterminer le déroulement de la pathogenèse chez les larves atteintes.

Des œufs récoltés en plein air ont été désinfectés en surface, puis placés en laboratoire sur un milieu nutritif à base de pollen. Les larves des 3^e et 4^e stades ont été transférées sur un milieu nutritif frais auquel on avait inoculé des spores d'*Ascosphaera aggregata*, l'agent responsable du couvain plâtré chez *M. rotundata*. On a fixé les larves à intervalles réguliers après inoculation, puis effectué des coupes à la paraffine, examinées ensuite au microscope optique et au microscope électronique à balayage.

Les spores d'*Ascosphaera aggregata* ont germé dans l'intestin moyen et pénétré dans l'hémocèle des larves du 3^e et 4^e stade, 2 jours après l'inoculation. L'invasion de l'épiderme, des trachées et des muscles s'est produite trois jours plus tard. Au moment de la mort, de 3 à 7 jours après l'inoculation, l'hémocèle était presque entièrement rempli de mycelium. Les signes externes de la maladie ne sont apparus que peu de temps avant la mort de l'hôte.

Le couvain plâtré chez l'abeille domestique, *Apis mellifica*, peut être causé par l'ingestion de spores d'*Ascosphaera apis*, mais peut aussi survenir à la suite de la germination des spores sur la cuticule extérieure et de leur pénétration ultérieure dans l'hémocèle. On a observé que les spores *A. aggregata* utilisées dans cette étude ne germaient qu'à l'intérieur de l'intestin moyen des larves hôtes. Le taux de germination *in vitro* le plus élevé est obtenu lorsque les spores sont placées en incubation sur un milieu nutritif en présence d'une concentration élevée de gaz carbonique. Le potentiel d'oxydo-réduction dans l'intestin des larves de *M. rotundata* indemnes est faible, mais on ne sait pas si cette condition est nécessaire à la germination *in vivo* des spores d'*A. aggregata*.

ZUSAMMENFASSUNG

PATHOGENESE DER KALKBRUT BEI DER LUZERNEN-BLATTSCHNEIDERBIENE, *MEGACHILE ROTUNDATA*

Kalkbrut ist eine Mykose der Larven von verschiedenen Hymenopteren, zu denen auch die Luzernen-Blattschneiderbiene, *Megachile rotundata*, gehört. Zur Untersuchung des Verlaufs der Pathogenese bei befallenen Larven wurden grundlegende Experimente durchgeführt.

Im Freien gesammelte Eier wurden auf der Oberfläche desinfiziert und im Labor auf pollenhaltige Nahrung gesetzt. Larven des 4. und 5. Stadiums wurden auf frische Nahrung gesetzt und mit Sporen von *Ascosphaera aggregata*, dem Verursacher der Kalkbrut bei der Luzernen-Blattschneiderbiene, beimpft. Nach der Fixierung der Larven in regelmäßigen Zeitabständen nach der Inoculation wurden Paraffinschnitte angefertigt und sowohl im Licht- wie auch im Rasterelektronenmikroskop ausgewertet.

Ascosphaera aggregata-Sporen keimten im Mitteldarm und penetrierten das Hämocoel des 4. Larvenstadiums bereits 2 Tage nach der Inoculation. Die Invasion der Epidermis, der Tracheen und Muskeln erfolgte innerhalb der drei folgenden Tage. Das gesamte Hämocoel war zur Zeit des Todes — 3 bis 7 Tage nach der Inoculation — angefüllt mit Myzel. Äußerliche Anzeichen für die Krankheit waren bis kurz vor dem Tod des Wirts nicht sichtbar.

Die Kalkbrut der Honigbiene, *Apis mellifera*, entsteht durch Aufnahme der Sporen von *Ascosphaera apis* durch die Bienenlarve, kann aber auch durch Sporenkeimung auf der Kutikula und anschließendes Eindringen in das Hämocoel hervorgerufen werden. Sporen von *A. aggregata*, die in diesen Untersuchungen verwendet wurden, keimten nur im Mitteldarm der Wirtslarve. Die höchste Keimrate *in vitro* wurde erzielt, wenn die Sporen auf einem Nährmedium bei erhöhter Kohlendioxidkonzentration

inkubiert wurden. Obgleich im Darm von nicht befallenen *M. rotundata*-Larven ein reduziertes Oxidations-Reduktions-Potential herrscht, ist nicht bekannt, ob dieses für die Keimung von *A. aggregata* Sporen *in vivo* notwendig ist.

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